Modification of oestrogen signalling during the acquisition of hormone resistance in breast cancer cells

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This is for my parents.

Für meine Eltern.

Declaration

In accordance with the regulations of the University, I declare that this thesis has been composed entirely by myself and that the work presented is my own except where I have indicated the contribution of others.

Barbara Kuske

July 2005

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Abstract

The oestrogen receptor (ER) mediates both normal and malignant breast development. The ER can exert its ligand-dependent gene regulatory functions directly by association with target genes and subsequent dynamic formation of a transcription complex through its oestrogen response element. The complex receptor-ligand signalling network has received much interest as a potential therapeutic target. Endocrine agents, such as tamoxifen, have been developed to reverse oestrogen (E_2) stimulated gene transcription and tumour growth, however progression to endocrine resistance provides a major obstacle in breast cancer treatment. This study investigates changes in endocrine response and ER transcription activation during the acquisition of endocrine resistance.

Breast cancer cell lines were selected that encompassed the range of oestrogen and anti-oestrogen sensitivities from the E_2-dependent and tamoxifen-sensitive MCF-7 cell line through the less sensitive LCC-1 and LCC-2 lines to the insensitive LCC-9 and independently derived LY2 line. All lines had been derived from MCF-7 cells. These models represent different endocrine phenotypes and were designed to reflect sequential changes in the clinical progression from hormone sensitive to hormone insensitive and antioestrogen resistant. MDA-MB-231 breast cancer cells were used as ERα negative controls. Growth assays confirmed these phenotypes and proliferative behaviour in response to E_2 and tamoxifen.

To help evaluate the role of the ERα in the development of endocrine resistance, quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) and Western blot analysis was carried out to assess transcriptional and translational receptor status for each phenotype. All resistant lines possessed greater levels of ERα mRNA than wild-type MCF-7 cells. E_2 downregulated ERα mRNA and protein. Results suggest ERα functionality in these cell lines. Analysis of mRNA levels of several ER target genes (pS2, progesterone receptor (PR), cathepsin D and MYC) indicated informative differences between lines. pS2 was highly expressed in resistant lines as was PR in most lines compared to MCF-7 cells. E_2 and tamoxifen produced attenuated or no effects in pS2 expression compared to the marked induction produced by E_2 in MCF-7 cells. Minor changes were also observed in the expression

levels of various coactivators (SRC-1, SRC-2, SRC-3) and corepressors (NCoR, SMRT and RIP140) investigated by qRT-PCR and Western blot.

A subset of three cell lines (MCF-7, LCC-1 and LCC-9) was used to examine transcription complex assembly at the pS2 promoter in response to E_2 utilizing chromatin immunoprecipitation. This identified a dynamic cycle of increasing H4 acetylation (indicative of active transcription) and ERα as well as cofactor recruitment upon E_2 addition in MCF-7 cells. A distinctive H4 acetylation pattern was revealed for LCC-1 and LCC-9 cells. While ERα recruitment was similar to MCF-7 cells, particularly strong SRC-1 and SRC-3 recruitment was detected in LCC-1 but most markedly in LCC-9 cells, implying altered pS2 transcription complex assembly.

The data suggest that a functional oestrogen receptor may remain in these endocrine resistant models. Cell proliferation and E_2 target gene expression in LCC-1 cells proved to be oestrogen and antioestrogen independent but responsive in the presence of the ligand whereas LCC-9 cells manifest complete endocrine resistance. In addition, changes have been detected in the assembly of a gene transcription complex at the pS2 promoter. Taken together, this is evidence for modified ER mediated transcription activity. These results may help to identify potential mechanisms of endocrine resistance.

List of most common abbreviations

aa:		Amino acid
AR:		Androgen receptor
Bp:		Base pairs
°C:		Degrees centrigrade
ChIP:		Chromatin Immunoprecipitation
CRUK:		Cancer Research UK
CTSD:		Cathepsin D
DCC:		Dextran activated charcoal stripped foetal calf serum
DMEM:	Complete	Dulbecco's Modified Eagle Medium (+phenol red) + 10% FCS serum + Antibiotics
	Reduced	Dulbecco's Modified Eagle Medium (-phenol red) + 5% dextran charcoal stripped serum + 1% glutamine + Antibiotics
DMSO:		Dimethyl sulphoxide
E_2:		17β-oestradiol
EGF:		Epidermal growth factor
ER:		Oestrogen Receptor
FCS:		Foetal calf serum
h:		Hour
HAT:		Histone acetylate transferase
HDAC:		Histone deacetylase
ICI:		ICI 182,780
mg:		Milligram
ml:		Millilitres(s)
MAPK:		Mitogen activated protein kinase
min:		Minute(s)
4-OHT:		4-hydroxytamoxifen
PBS:		Phosphate buffered saline
PCR:		Polymerase Chain Reaction
PR:		Progesterone Receptor
RAL:		Raloxifene

RAR:	Retinoid Acid Receptor
RPM:	Revolutions per minute
RT:	Room temperature
RT-PCR:	Reverse Transcriptase-Polymerase Chain Reaction
TAM:	Tamoxifen
TGFα:	Transforming growth factor α
TOT:	*trans*-hydrotamoxifen
WT:	Wild type

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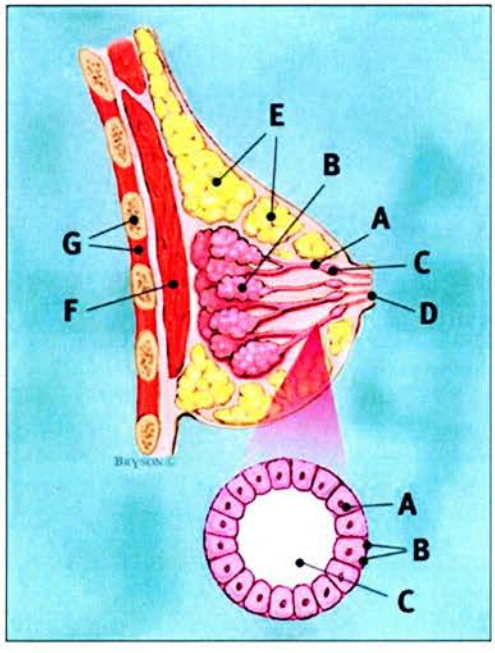
Chapter 1: Introduction

1.1 Breast Cancer

Breast cancer is a common disease with 1 in 9 woman in the UK developing this illness during their lifetime. It is a disease with a high level of public awareness and one of the best-funded areas of research in the world. Remarkable insights into the biology of this cancer have been gained and significant progress has been made in terms of treatment and outcome. However, many patients become resistant to treatment or never respond at the outset. 'Further research is needed' is the phrase included in many publications summarizing the underlying mystery that still surrounds this disease.

1.1.1 Breast cancer classification

The normal female breast develops fully between the ages of 12 and 19 years. It consists of lobules or glands grouped together into 15-20 lobes. Ducts emanate from each lobule forming a lactiferous duct which merge beneath the nipple to form a lactiferous sinus. Milk is secreted during pregnancy in the terminal duct lobular unit in response to the hormone oxytocin. The breast also consists of adipose and connective tissue (see figure 1.1).



Breast profile:

A duct system
B lobules
C dilated section of duct to hold milk (lactiferous duct)
D nipple
E adipose tissue
F pectoralis major muscle
G chest wall/ rib cage

Enlargement:

A normal ductular epithelial cells
B basement membrane
C lumen (center of the duct)

Figure 1.1: Schematic representation of the female breast (Source: www.breastcancer.org).

Breast cancers can be classified as either in situ (non-invasive) or invasive cancers (Sainsbury, J.R.C. *et al.*, 2000). Most cancers develop in the lining of the ducts but they can also arise in the lobules or the stroma. The most common form of pre-invasive breast cancer, ductal carcinoma in situ (DCIS), evolves only in the milk ducts while invasive ductal carcinoma (IDC) spreads through the duct walls. Similar classifications are made for lobular carcinoma in situ and invasive lobular carcinoma. Less common forms of breast cancer include inflammatory cancers or medullary carcinomas (which originates in the central breast tissue). Figure 1.2 shows a mammogram of a normal breast and a mammogram of a breast showing an abnormality later confirmed to be a malignant lesion.

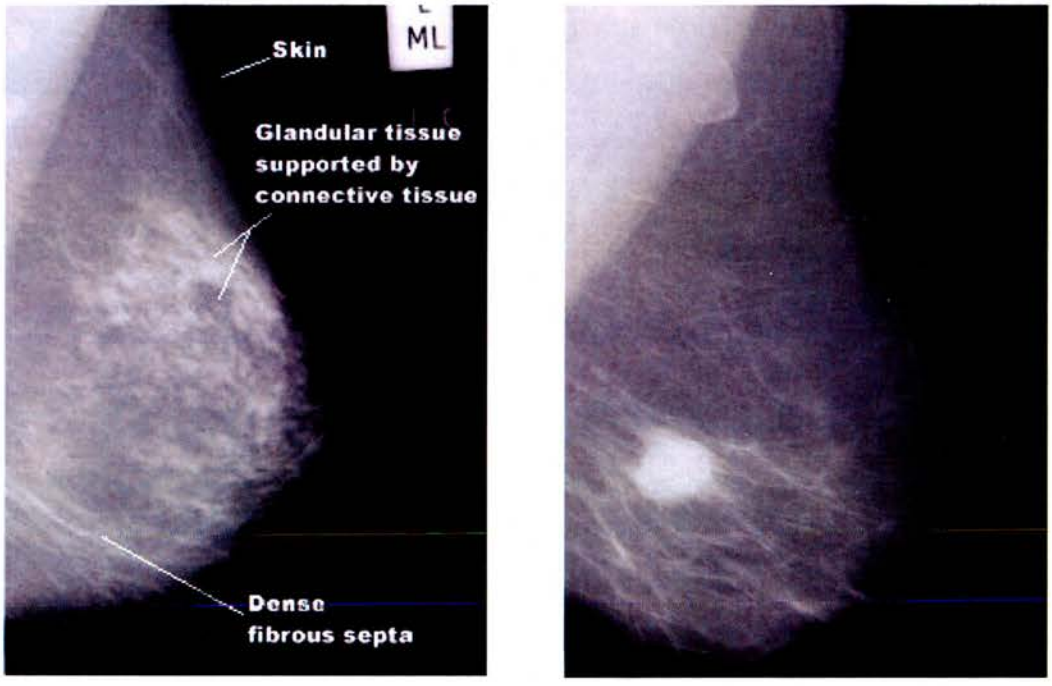


Figure 1.2: Mammogram of a normal breast (left) and a breast showing an abnormality (Source: Interactive Mammography Web Tutorial, McGill University Faculty of Medicine).

1.1.2 Incidence, mortality and epidemiology

(i) Worldwide

There are an estimated 10 million new cases of cancer occurring each year (Parkin D.M. *et al.* 2001 (a), (b) and(c)). Breast cancer, with 1.1million (10.4%) cases, is the second most common cancer overall after lung cancer (12.3%) and remains by far the most common cancer in woman. However, the disease only ranks

fifth with 370 000 deaths (6%) of 6.2 million death caused by cancer. This favourable trend is considered to be due to improving treatment options compared to lung or stomach cancer. Age standardized incidence and mortality rates for the disease vary greatly between different countries. Generally high rates of incidence can be found in Northern America, Europe and Australia (GLOBOCAN 2000 in Parkin, D.M. *et al.*, (a)), which indicates a strong link between more affluent areas and high numbers of breast cancer cases.

(ii) In the UK

In the UK, 41,000 new cases of breast cancer and 13,000 deaths due to the disease occur every year. The number of new cases accounts for more than 1 in 4 of all cancers diagnosed in woman. Over the course of the last 25 years and up to 2000, the UK has seen a steady increase of age standardized female breast cancer incidence rates rising from 79 to 114 per 100 000 women. This increase can partially be attributed to more frequent screening and more thorough national case registration over these years. The lifetime risk of developing breast cancer in the UK is 1 in 9 across all ages. The age-standardized 5 year relative survival rate (taking into account the underlying rates of death in woman of the same age) has increased from about 52% between 1971-1975 to about 77% between 1996-1999 as documented in table 1.1 (Cancer Research UK (CRUK), 2004).

1971-1975	**1976-1980**	**1981-1985**	**1986-1990**	**1991-1995**	**1996-1999**
52%	59%	62%	67%	73%	77%

Table 1.1: Age standardized 5-year survival rates for female breast cancer patient's diagnoses 1971-1999, England and Wales (Source: http://www.cancerresearchuk.org/aboutcancer/statistics/statsmisc/pdfs/factsheet_breast_feb2004.pdf).

1.1.3 Aetiology

Most risk factors associated with a greater chance of developing breast cancer are the consequence of higher exogenous or endogenous oestrogen levels in the cell consistent with oestrogen acting as a promoter to stimulate tumour growth.

(i) Age

The strongest risk factor for breast cancer is age. The incidence of breast cancer in woman increases considerably up until menopause. In the UK in 2000, 587 new cases were diagnosed at the ages of 30-34 years compared to 5586 new cases between the ages of 50-54 years (CRUK, 2004). Once menopause is reached the rate of increase in developing the disease slows down. Breast cancer under the age of 30 is rare (CRUK, 2004).

(ii) Age at menarche and menopause

Early onset menarche as well as late onset of menopause have both long been related to an increased risk of breast cancer. Woman who reach menopause after the age of 54 years are subject to a twofold increase in the risk of breast cancer. A threefold increase is likely for women who reach menarche before the age of 11 years (McPherson, K *et al.*, 2000). Both factors could be based on prolonged exposure to endogenous oestrogen during regular ovulatory cycles (Parl, F.F., 2000).

(iii) Family history

A genetic predisposition is widely acknowledged as a risk factor in this disease. Hereditary factors account for an estimated 5-10% of all breast cancers (Ellison L.W., *et al.*, 1998). Several genes have been demonstrated to be linked with susceptibility to breast cancer including tumour suppressor genes BRCA1 and BRCA2 located on chromosome 17q21 and 13q12-13, respectively. Women carrying mutations of BRCA1 and BRCA2 are estimated to have an 85% lifetime risk of developing breast cancer. Mutations occur at almost any position (McPherson K. *et al.*, 2000; Dumitrescu, R.G. and Cotarla, I. 2005). Complete loss of function for both genes is a result of carriers exhibiting the loss of the wild-type allele. There is a very high probability for this loss, when carrying a germ line mutation in one of the two alleles of the gene. BRCA1 and BRCA2 are considered 'caretaker genes', one of two groups of cancer susceptibilty genes responsible for maintaining genomic integrity (reviewed in Venkitamaran, A.R. *et al.* 2002). Mutations of these genes causes accumulation of altered 'gatekeeper genes', the gene group whose changed functions alters regulation of cell cycling, cell proliferation and cell death.

Much effort has been spent on determining how wild-type BRCA proteins play a role in preserving chromosome structure. BRCA1 function has been linked to several aspects of DNA damage repair as well as the regulation of transcriptional events (reviewed in Wang, Q. 2000). For example, co-localization has been reported with DNA repair and recombination protein RAD50 or RAD51 protein complexes, suggesting that BRCA1 might be directly involved in repair-work of double-stranded DNA breaks (Scully, R. *et al.* 1997(a) and (b); Zhang, H. *et al.* 1999). BRCA1 has been shown to associate with chromatin remodelling complexes containing HDAC as well as CBP/p300 proteins to modulate gene transcription in breast and ovarian cancer (Yarden, R.I. and Brody, L.C. 1999; Pao, G.M. *et al.* 2000). BRCA1 and BRCA2 genes have been suggested to play a role in cell cycle control with one or both proteins able to regulate cell cycle arrest and/or apoptosis (reviewed in Venkitamaran, A.R. *et al.* 2002). Most mutations linked to breast cancer show little evidence to be familiar rates of mutations but are in fact sporadic forms, for example mutations found on the tumour suppressor genes p53 and PTEN/MMAC1 (reviewed in Feki, A. and Irminger-Finger, I. 2004; de Jong, M.M. *et al.* 2002). p53 is located on chromosome 17p13.1 and is thought to play a role in DNA repair as well as prevention of replication of damaged DNA. Mutations of p53 are common and identified in approximately 50% of all human cancers including breast, ovarian and lung cancer. The risk of developing breast cancer for women carrying a p53 mutation decreases with age but is estimated to be 18 fold higher compared to the general population.

(iv) Life style

Much debate has been sparked by the fact that breast cancer incidence is much higher in Western European countries or the US than African or Asian populations (Parkin, D.M. *et al.* 2001). This is thought to be due to geographical as well as lifestyle factors such as income, education or housing which are linked to a higher incidence of breast cancer. For postmenopausal woman, being overweight increases the risk of breast cancer by around 50% most likely by increasing the exposure to endogenous oestrogen (reviewed in Key, T.J. *et al.*, 2002). However, this does not appear true for pre-menopausal woman where obesity is associated with a slight decrease in breast cancer incidence (reviewed in Hankinson, S.E. *et al.* 2004).

As with early pregnancies and breastfeeding in younger women, these factors decrease the risk longterm as the number of ovalutory cycles and therefore the levels of endogenous oestrogen are reduced. Also, an increased alcohol intake might be associated with an increased risk for breast cancer (Petri, A.L. *et al.* 2004, and others). The mechanisms for this association are unknown but might include the hypothesis that alcohol induces genome instabilities such as chromosome 17 aneuploidy (Benassi B. and Fenech M., 2004) or increases in oestrogen concentration in the serum (Dorgan, J.F. *et al.* 2001). Taken together, evidence for any dietary or other lifestyle risk factors remains controversial and their effects are likely to alter the balance of circulating oestrogen and other hormones (Key, T.J. *et al.*, 2002).

(iv) Hormone replacement therapy

When analysing evidence for the risk of breast cancer as a result of hormone replacement therapy (HRT), a clear distinction is made between current and never or no-longer users as well as long-term and short-term users. In general, the incidence risk increases most significantly for women who are currently and have been using HRT for more than 5 years. A study by the Collaborative Group on Hormonal Factors in Breast Cancer (Collaborative, 1997) which included 51 studies in 21 countries demonstrated that the relative risk of being diagnosed with breast cancer for woman currently using or having used 1-4 years previously, increases by a factor of 1.023 per year of use. The risk for woman having used HRT for 5 years or longer is increased by a factor of 1.35. Interestingly, this increase diminishes when HRT usage has been stopped for more than five years. The risk for women who never have or have ceased using HRT is reduced or disappears. One of the largest studies to demonstrate the increased risk for current HRT users to develop and die from breast cancer for was the so called 'Million Women Study' (Beral, V. *et al.* 2003). This study also established that continuous HRT combining oestrogen with progestagens carries a greater risk than other HRT combinations or oestrogen alone (Beral, V. *et al.* 2003; Tjonneland, A. *et al.* 2004). When evaluating the HRT associated breast cancer risk, it has to be taken into account that combined HRT can decrease sensitivity for mammography. Combined oestrogens and progestogens can increase breast density, a fact that reflects negatively on the efficacy of breast screening.

Risk factor	High risk group
Gender	Female
Age	Elderly
Genetic predisposition	BRCA1/2 mutation carriers
Geographical location	Developed country
Family history	BC in first degree maternal relative
Reproductive span	Early age at menarche Late age at menopause
Age of pregnancy	Late age at first pregnancy
Parity	Null parity
Previous benign disease	Atypical hyperplasia
Endogenous hormones	Current use of oral contraceptives Long-term hormone replacement therapy
Height	Tall individuals
Diet	High fat intake
Benefit factors:	Physical activity
	Long duration of lactation

Table 1.2: Possible risk factors for breast cancers as suggested by a literature review.

(Dixon, J.M. 2003). A certain number of breast cancers might therefore remain undetected or will not be be detected until later stages. Overall, interpretation of results in these studies, regarding the effects of HRT on breast cancer, is difficult. It is often commented that, particularly in large cohort studies, clear subgroups of HRT users and information on the length and the hormone combination is not consistently known. In addition, when comparing breast cancer incidences, HRT users are more likely to be seen regularly by a physician than non-users, which might increase their chance of being diagnosed with breast cancer. Known and suspected risk factors contributing to an increased risk of breast cancer as reviewed in the literature are summarized in table 1.2.

1.1.4 Screening and diagnosis of breast cancer

Breast cancer screening aims to detect any malignancy as early as possible and before any spread has occurred to increase the potential for a cure. Many women detect palpable malignancies by self-assessment. However, the most effective screening method is mammography particularly for women aged 50-70 years which can reduce mortality from breast cancer for up to 40% in those who

attend (Blamey, R.M. *et al.*, 2000). The National Breast Cancer Screening Programme in the UK currently advises every woman between the age of 50 and 64 years to take part in mammographic screening every 3 years. The program is being extended to include women up to the age of 70 years in 2004 nationwide. Mammography can detect palpable as well as non-palpable abnormalities since carcinomas generally display a greater density than the surrounding tissue. If an abnormality has been detected or is suspected by mammography, a tissue sample can be extracted for further investigation by the cytopathologist. Fine needle aspiration or core biopsies remove smaller lesions or draw tissue samples from the affected area which can then be examined for the presence of malignancy and other diagnostic factors such as their ER/ PR status determined through immunoperoxidase staining. The tumour is staged and graded as described in the next section and a treatment plan is established based on the combined results of these diagnostic procedures.

In addition to the primary detection methods, genetic testing is offered for women who are at a particularly high risk of developing breast cancer. Among these are women who have had several cases of breast cancer within the family, especially on the same side. Familial breast cancer is also suspected when cancers occur at younger ages or other forms of cancer have evolved such as ovarian or bowel cancer, cancers that have shown to be linked to the occurrence of breast cancer.

1.1.5 Treatment of breast cancer

(i) Staging and grading of breast cancer

The choice of treatment for breast cancer is strongly influenced by the stage and grade of the tumour as well as the overall health and the wishes of the patient. The stage of a breast cancer categorizes tumours according to their size and degree of spread where stages I-III are considered primary breast cancers with possible regional (nodal) metastases and stage IV implies secondary cancers, tumours of any size with involvement of distant metastases (see table 1.3). Grading breast cancer is based on the histology of the tumour. Grade 1 to 3, corresponding with high to lower survival rates, are assigned using histological characteristics such as tubule formation or mitotic counts of the carcinoma.

Stage	Tumour diameter	Involvement of regional spread	Evidence of distant spread
I	2cm or less	no	no
II	2-5cm	Yes (lymph nodes)	no
II	5cm or greater	no	no
III	5cm or greater	Yes (skin, lymph nodes, pectoral and chest wall fixation)	no
IV	Any size	Yes or no	yes

Table 1.3: Staging system for breast cancer tumours.

(ii) Chemotherapy

The systemic forms of breast cancer treatment used are either neoadjuvant (before surgery) or adjuvant (supplemental, after surgery). Neoadjuvant chemotherapy is given to reduce tumour size and determine the tumours response to this form of treatment for subsequent treatment. Adjuvant chemotherapy is given in addition to other forms of treatment such as surgery to reduce the chances of cancer recurrence by eliminating metastatic forms of the disease. Chemotherapy is administered either orally or, more commonly, intravenously in cycles (administration over several days followed by 3-4 week breaks) and can last up to 6 month (Cancer Research UK Website). The most commonly used drugs include cyclophosphamide (cytoxan), Epirubicin (Ellence), 5-Fluorouracil (5 FU/Adrucil) or Methotrexate (Amethopterin/ Mexate/ Folex). Drugs are often used in combination to maximize efficacy. Most common combinations include CMF (cyclophosphamide, Methotrexate, 5-Fluorouracil) and FEC (5-Fluorouracil, Epirubicin, cyclophosphamide). The nature of such systemic therapy leads to a variety of side effects experienced by patients. More temporary side effects include nausea and vomiting, hair loss, fatigue and anaemia while potential permanent effects include premature menopause and infertility (CRUK Website; Imaginis: The Breast Health Resource Website).

(iii) Surgery

Most patients are ultimately offered surgical procedures to remove carcinomas and obtain a lymph node sampling. Surgery can involve removing localized carcinomas (lumpectomy) or the removal of the carcinoma, surrounding affected tissue or lining of the chest muscle (partial mastectomy). The most common form of surgery involves the removal of the breast in addition to lymph nodes and affected chest wall lining or muscles (modified radical mastectomy). Surgery is often combined with internal or external radiation therapy or chemotherapy to eliminate any remaining cancerous cells and reduce the chances of recurrence (see 1.4 (ii)).

(iv) Hormone therapy

Hormone therapy is primarily used for oestrogen and progesterone receptor positive cancers. Various endocrine agents are designed to reverse oestrogen stimulated tumour growth in different ways. They can be divided into two major groups: (a) *'Classical' antioestrogens*: Triphenylethylene derivatives such as tamoxifen and other non- steroidal compounds such as 'Fixed ring' structure Raloxifene; and the more recently developed (b) *Steroidal and non-steroidal aromatase inhibitors* such as Letrozole.

Tamoxifen, an oestrogen derivative, with its relatively low toxicity has proven to be a significant part of breast cancer chemoprevention and treatment (Clarke, R. *et al.* 2003) (figure 1.3). It is generally associated with an increase in disease free and overall survival and used long term for up to 5 years in adjuvant therapy. Tamoxifen prevents oestrogen stimulated gene transcription by competition with oestrogen for ER binding. However, many tumours eventually grow resistant to this antioestrogen. Tamoxifen's known partially agonistic effects also result in unwelcome side effects. This lead to the development of other strategies including anti-oestrogenic agents ICI 182,780 (Fulvestrant/Faslodex) and ICI164,384. Oestradiol analogues are steroidal ER inhibitors regarded as pure antagonists because of their lack of oestrogenic activity (reviewed in Osborne, K. *et al.* 2000). ICI 182,780 is primarily used in advanced forms of the disease after tamoxifen failure and has shown to use a different molecular mechanism to block oestrogen binding to

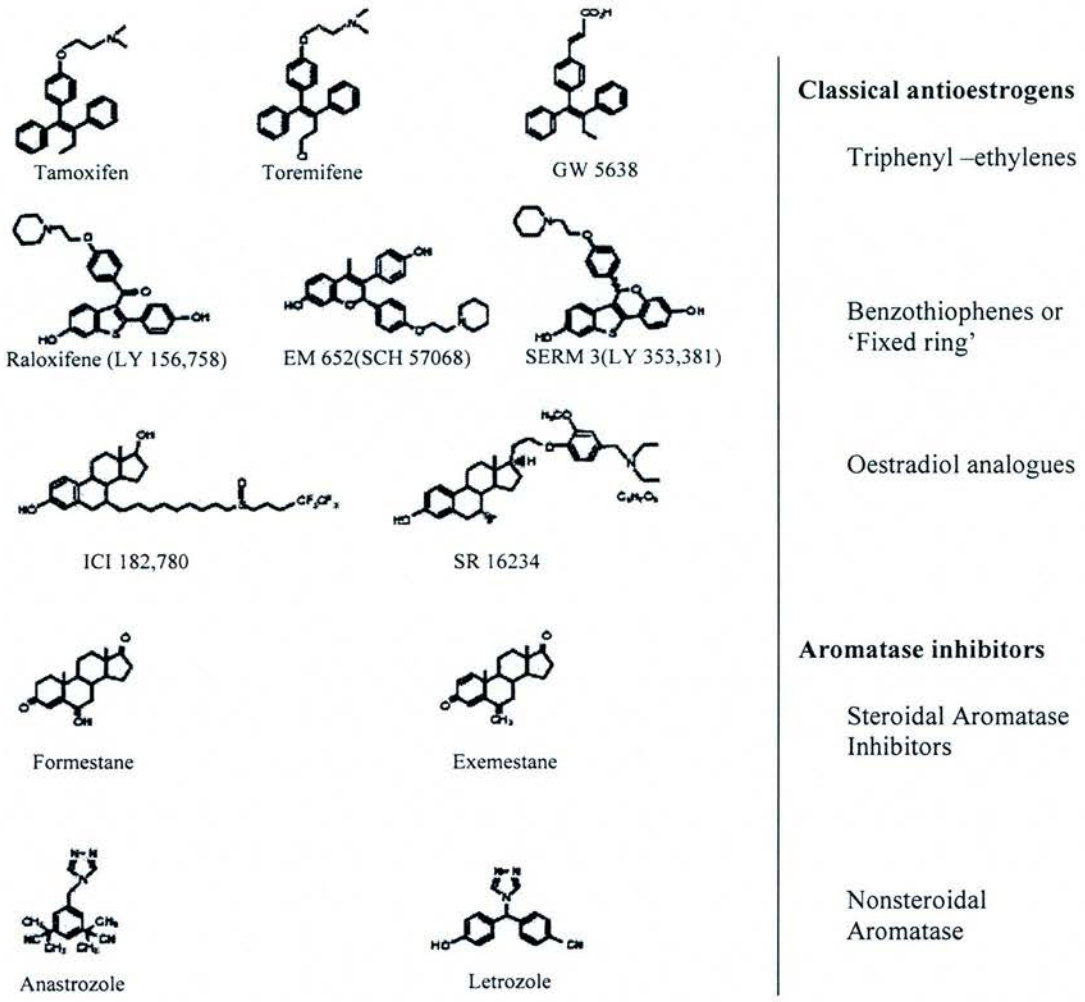


Figure 1.3: Chemical structure of selected 'classical' antioestrogens and aromatase inhibitors (source: Howell, A. *et al.* 2001).

the receptor which might avoid cross-resistance to tamoxifen. ((Howell, A. *et al.* 2001; Gradishar, W.J., 2004). However, purely antioestrogenic also implies therapeutic disadvantages as the use of these agents increases bone loss. The mechanism of action of antioestrogens as well as the antioestrogen resistance is discussed in more detail in chapters 1.4.1 and 1.4.2.

Benzothiophenes, such as Raloxifene (keoxifene, LY 156,758), are structurally similar to triphenyl-ethylenes. Both groups are non-steroidal antioestrogens and considered competitive ER inhibitors with partial agonistic activities. The three-dimensional structure of Raloxifene inlcudes a 'fixed ring'. Raloxifene significantly reduced the risk of ER positive breast cancer development in

postmenopausal women (Cauley, J.A. *et al.* 2001). Compared to tamoxifen, it showed substantially less oestrogenic activity in the endometrium (Cummings, S.R. *et al.* 1999). Raloxifene also shows beneficial effects on bone density and was further developed for the prevention and treatment of osteoporosis (Delmas, P.D. *et al.* 1997).

A more recent option is aromatase inhibitors such as anastrazole or letrozole offering a more potent and better tolerated form of treatment to postmenopausal, hormone sensitive breast cancer. Unlike antioestrogens, aromatase inhibitors prevent the conversion of androgens to oestrogens actively removing a primary source of oestrogens available to the receptor. In the so-called Anastrazole, Tamoxifen Alone or in Combination (ATAC) trial, the largest of its kind so far, anastrazole, as adjuvant treatment for postmenopausal woman, demonstrated enhanced efficacy as progression-free survival time was increased by 19% and the incidence for new contra-lateral tumours decreased by 58% compared with tamoxifen (Buzdar, A.U.; ATAC Trialists' Group, 2003 and Dowsett, M. and Howell, A. 2002). In addition, patients seem to tolerate treatment with anastrazole generally much better although nausea and muscoskeletal pain are common side effects. Results of these studies are likely to lead to the use of aromatase inhibitors as the preferred choice of therapy in early breast cancer although the use of agents in sequence for the optimal treatment might always include antioestrogens. The continuous withdrawal of oestrogen is likely to cause adverse effects on other systems such as bone metabolism and cardiovascular functions (Dowsett, M. and Howell, A. 2002; Haynes, B.P. *et al.* 2003). The ATAC trial showed that patients receiving anastrazole have a greater incidence of bone fractures reflecting a possible increase in bone mineral loss (ATAC Trialists' Group, 2002). Letrozole has shown to increase urinary and plasma markers of bone resorption (Heshmati, H.M. *et al.* 2002; Harper-Wynne, C. *et al.* 2002). Further research assessing long-term effects of aromatase inhibitor is needed to ensure the agents beneficial effects outweigh the side effects.

Aside from antioestrogens and aromatase inhibitors, pituitary down regulators (such as 'goserelin'), ovariectomy or radiotherapy targeting the ovaries are sometimes considered in pre-menopausal woman as strategies to suppress hormone production and reduce exposure to endogenous levels of oestrogen.

(v) Other forms of treatment

A more recently developed form of breast cancer treatment is a monoclonal IgG1 class- humanized antibody Herceptin (trastuzumab) designed to the extracellular domain of the HER2/neu receptor. The HER2/neu receptor is a member of EGFR/HER/erb family of transmembrane receptor tyrosine kinases which also includes the epidermal growth factor receptor HER1 (EGFR, erbB1), HER3 (erbB3) and HER4 (ErbB4)(figure 1.4)(Arteaga, C.L. 2003; Ross, J.S. *et al.* 2004). HER receptors are involved in signal transduction via phosphorylation and dephosphorylation of transmembrane proteins or signalling intermediates of several pathways such as the Ras/mitogen activated protein kinase (MAPK) or

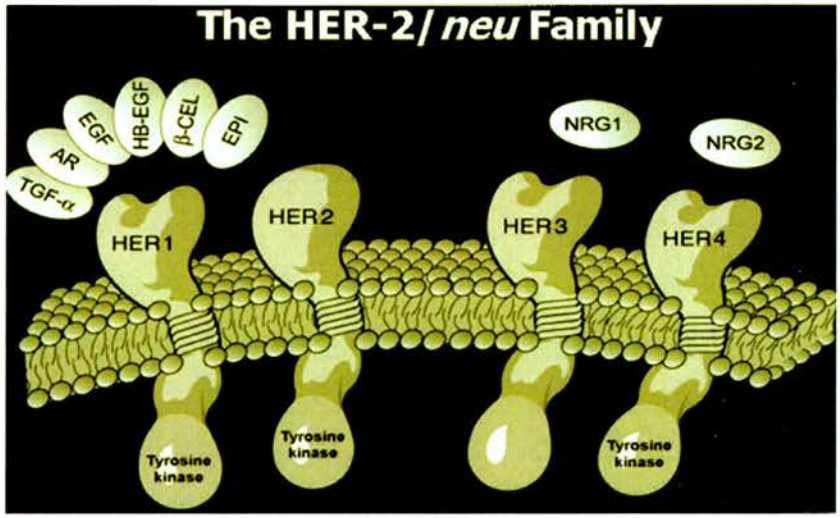


Figure 1.4: The HER2/ neu family of transmembrane receptor tyrosine kinases (source: Ross, J.S. *et al.* 2004)

phosphatidylinositol 3-kinase (p13K)/ Akt pathways which affect cell proliferation, survival, motility and adhesion. Activation of HER receptors follows ligand binding and dimerization with another receptor of the family. HER2 is the only receptor for which there is no known ligand, however, it is the preferred binding partner providing the most stable and potent combination of dimers (Tzahar, E. *et al.* 1996; Craven, R.J. *et al.* 2003).

HER2 has been found to be overexpressed in 20-30% of breast cancers mostly the result of amplification of the HER-2 gene (Slamon, D.J. *et al.* 1987; Pauletti, G. *et al.* 1996). The overexpression of HER-2 has been positively correlated

with a poor breast cancer prognosis involving several parameters such as disease recurrence, metastasis, short term survival and resistance to chemotherapy and hormone therapy (reviewed in Harries, M. and Smith, I. 2002). *In vitro* and *in vivo* studies have shown that herceptin has a significant antiproliferative effect in HER-2 overexpressing breast cancer, however, the mechanism of action remains elusive (Harries, M. and Smith, I. 2002; Ross, J.S. *et al.* 2004). It is likely that the mechanism involves receptor degradation preventing HER2/neu dimerization or a pro-apoptotic effect inhibiting PI3K/Akt signalling (De Santes, K. *et al.* 1992; Yakes, F.M. 2002). Herceptin is most widely used as a first- and second-line agent in HER-2 overexpressing metastatic breast cancer. It is used as a single agent or in combination with chemotherapeutic drugs such as paclitaxel (Arteaga, C.L. 2003; Ross, J.S. *et al.* 2004).

1.2 Oestrogens, Oestrogen Receptor and Breast Cancer

Oestrogen is classically defined as one of the natural steroids, a female sex hormone responsible for the stimulation of the development of female secondary sex characteristics and the promotion of the growth and maintenance of the female reproductive system. Oestrogens also play a role elsewhere in the human body, for example the male reproductive system, the cardiovascular and immune systems and in the development of diseases such as colon cancer and arthritis. The role of oestrogen in the development of breast cancer is complex and the subject of diverse intensive research.

1.2.2 Oestrogen synthesis and metabolism

All oestrogen molecules are a product of cholesterol metabolism and consist of an aromatic ring, a phenol hydroxyl group and a methyl group. In premenopausal women most oestrogen is biosynthesised in the theca and granulosa cells of the ovaries. This hormone synthesis is known as the 'two-cell' concept. Androgenic precursors, testosterone and androstenedione, originate in a multistep enzymatic reaction in the theca interna cells and are then aromatised into oestradiol (E_2) and estrone (E_1) in the granulosa cells. The synthesis of progesterone and testosterone is mediated by the luteinizing hormone (LH), the aromatisation to oestradiol and

estrone by the follicle-stimulating hormone (FSH). Hormone synthesis also takes place in the adrenal cortex and peripheral tissue, and adipose tissue. Oestrogen levels are maintained in the breast tissue by hormone uptake from the serum and local synthesis (Miller, W.R. and O'Neill, JS. 1987; Parl, F.F. 2000 (b)).

Once women reach menopause, non-ovarian oestrogen production is predominantly responsible for the maintenance of hormone levels in breast tissue. The steroids androstenedione and estrone sulfate circulate in an inactive form in breast tissue and are both converted to estrone by P450 aromatase and estrone sulphatase (E_1-STS), respectively. Then, 17β- hydroxysteroid dehydrogenase (17β-

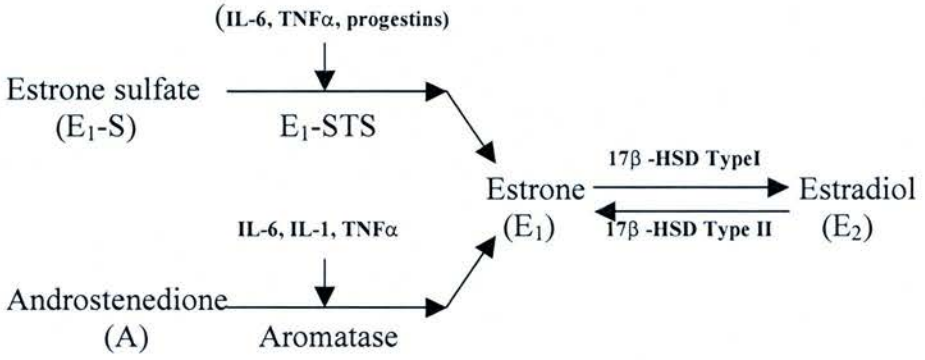


Figure 1.5: Oestradiol synthesis in breast tissue (Estrone sulfatase (E_1 –STS), Interleukin-1/6 (IL-1/6), Tumour-Necrosis Factor α (TNF).

HSD) converts estrone into the biologically active estradiol (see figure 1.5). Aromatase is encoded by the CYP19 gene. The gene contains multiple promoters. The expression of CYP19 is thought to be influenced primarily by promoter I.4 stimulated by cytokines such as IL-6 and TNF-α in normal tissue (Purohit, A. *et al.* 2002, Suzuki, T. *et al.* 2003). However, in malignant tissue, expression is mainly stimulated by promoter I.3 and II. Its regulation remains unclear but might be influenced by cAMP and Prostaglandin E_2 (PGE_2) (Purohit, A. *et al.* 2002).

The proposal of a local synthesis of oestrogen is based on the observation that after menopause there is a change in the ratio of E_2 concentration in plasma circulation compared to the concentration in the breast tissue. After menopause,

oestrogen levels decrease in the circulating serum but remain the same in malignant breast tissue (van Landeghem, AA *et al.* 1985). Hence oestrogen levels are actually much higher in the tumour compared to the level in the serum but also compared to normal surrounding tissue as summarized by Pasqualini, J.R. (2004). The local oestrogen source is the key in the development of newer treatment forms for breast cancer using aromatase inhibitors. The development of breast cancer is dependent on increased oestrogen exposure. The blockade of this synthesis pathway is thought to prohibit tumour progression in postmenopausal women. Not unexpectedly, even complete aromatase inhibition does not withdraw all oestrogen from the breast tissue and plasma. Alternative pathways must be available to synthesize oestradiol locally under the influence of enzymes such as STS and 17β-HSD Type1 or oestrogens entering through the diet.

1.2.3 Oestrogen receptor

Oestrogen mediates its actions by binding to the oestrogen receptor, a member of a large protein family consisting of nuclear receptors for vitamin A, testosterone and progesterone as well as orphan receptors to which specific ligands have not yet been identified.

(i) Oestrogen Receptor Structure

The oestrogen receptor α (ERα) gene is a 140kb gene located at 6q25.1 (Gosden, J.R. *et al.* 1986). It encodes a 595aa protein expressed primarily in the uterus, the liver, the kidney and the heart. A second oestrogen receptor gene, ERβ, is located at 14q22-24 and spans about 40kb (Enmark, E. *et al.* 1997). The ERβ protein consists of 485aa and is primarily found in the ovary, the prostate, lung,

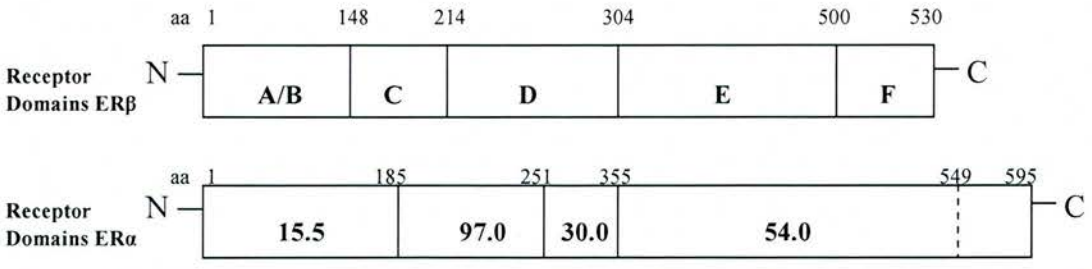


Figure 1.6: Percentage amino acid identiy between human ERβ and human ERα. Numbers above note amino acid position within each protein. Numbers within each box refer to percentage amino acid identity (adapted from: Enmark, E. and Gustafsson, J.-Å. 1999).

gastrointestinal tract, bladder, haematopoietic and central nervous systems. In addition, both receptor forms are also found in certain areas of the brain, the mammary gland, the thyroid and the bone. The human ERβ shows about 47% identity overall to the human ERα in its translated region (Enmark, E. and Gustafsson, J.-Å. 1999)(Figure 1.6). Exact amino acid positions as well as the degree of amino acid homology vary slightly between publications (figure 1.7).

Both steroid receptors consist of six main functional domains but with varying homology. The N-terminal domains A and B encode one of the two-ligand binding domains (LBD), activation domain (AF-1) associated with ligand - independent (constitutive) activity involved in protein-protein interaction and transcriptional activation of oestrogen target genes (figure 1.7). Here one of the striking functional differences between the two-receptor forms can be found reflected in the low degree of homology of only about 16% within the N-terminal region. In ERα, oestrogen mediates transcription of several oestrogen responsive genes by activation of the AF-1 domain but fails to have an impact on this domain in ERβ. Furthermore, two separate regions within the AF-1 domain of ERα, which are not found in ERβ, are necessary for the agonistic effect of oestradiol. Synthetic antioestrogens such as tamoxifen or raloxifene show a partial agonistic effect as opposed to a pure antagonistic effect in ERβ (reviewed by Nilsson, S. *et al.* 2001). This might be one functional variation contributing to the different response of specific tissues to oestrogens and antioestrogens. The highest degree of homology between the two receptors with 97% identity is observed at the

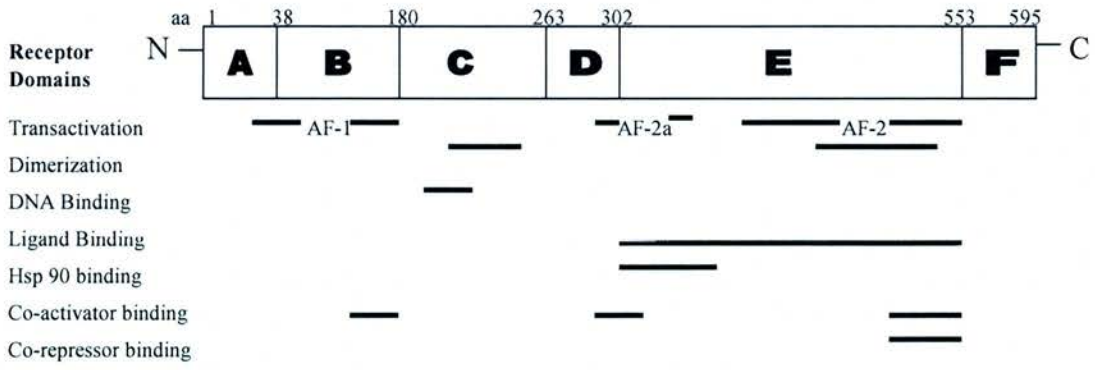


Figure 1.7: Schematic diagram of oestrogen receptor α protein and its structural and functional domains. Numbers note amino acid positions within protein. Note: Exact amino acid positions vary between publications (figure adapted from Sommer S. and Fuqua, S.A.W., 2001).

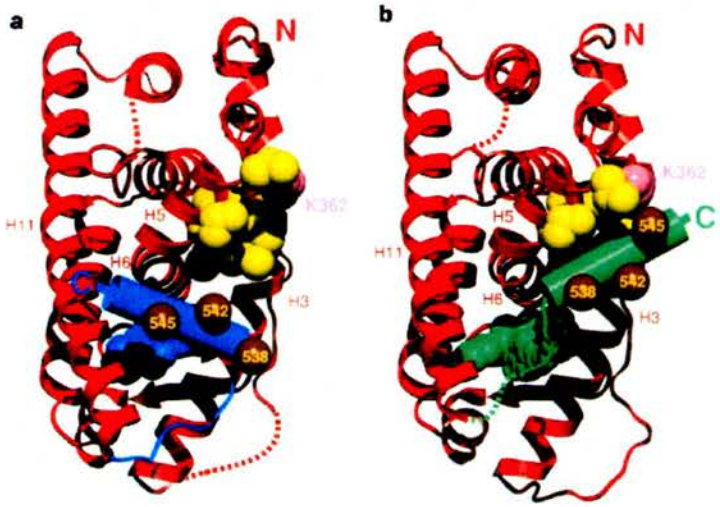


Figure 1.8: Positioning of helix H12. **a:** ER LBD–E2 complex, **b:** ERLBD–RAL complex.
H12 is drawn as a cylinder and coloured blue (E2 complex) or green (RAL complex). The remainder of the ER LBD is shown in red.Dotted lines indicate unmodelled regions of the structures. Hydrophobic residues located in the groove between H3 and H5 (yellow) and Lys 362 (K362, pink) are depicted in space-filling form. The locations of Asp 538, Glu 542 and Asp 545 are highlighted (brown spheres) along with the helices that interact with H12 in the two complexes (source: Brzozowski, A.M. *et al.*1997).

DNA binding domain (DBD), the C-domain of the receptor, with only two aa differing (reviewed in Parl, FF (c) 2000). This domain contains two zinc fingers to mediate conformational changes in the receptor and the binding of the receptor to the promoter of the ER responsive gene. As a result of the structural resemblance, receptor binding shows similar specificity and affinity for the ERE binding to the promoter. Regions D-F at the COOH-terminal are the most important for ligand binding, receptor dimerization and nuclear translocation. This region includes a ligand-binding pocket, which harbours the functionally critical activation domain AF-2. Crystallographic structures have revealed that the LBD contains an interacting surface composed of aa spanning helices 3-12 (Parl, FF (c) 2000). The AF-2 region stretches over helices 3, 4, 5 and 12 where helix 12 (H12) is specifically altered depending on the ligand bound to the receptor (figure 1.8) (Brzozowski, A.M. *et al.*1997; Shiau, A.K. *et al.* 1998). When agonists oestradiol or diethylstilbestrol (DES) are bound to the LBD, H12 positions itself in a way that seals the binding cavity, generating an interacting surface for coactivators necessary for the transcription initiation of target genes. In contrast, when antioestrogens such as raloxifene or tamoxifen bind to the LBD, their long side chains prevent H12 from

covering the binding cavity as with oestradiol binding. Instead, H12 occupies helices 3,4 and 5 while blocking the coactivator binding sites at H12 and preventing the activation of transcription. Distinct receptor conformations caused by different ligands and specific H12 positioning serve as molecular evidence for agonistic or antagonistic response. Such conformational changes have been observed for liganded states at the activation domains in both ERα and ERβ (Pike, A.C. *et al.* 1999).

(ii) Oestrogen receptor isoforms/ splice variants

There is an abundance of splice variants that have been identified for the ER receptor including mRNA variants with duplications, insertions or deletions of alternative exons at either the NH_2 or the COOH terminus, the DNA or ligand-binding domains. The nomenclature and classification for the identified isoforms varies to a great extent. Here, non-coding region and coding region variants will be differentiated. The same nomenclature as suggested by Flouriot, G. *et al.* (1998) is used (detailed review of ER mutations by Herynk, M.H. *et al.* (2004). The described mRNA isoforms described are all products of non -mutated genes. Other variants generated from genes altered by nucleotide substitutions and deletions have also been identified.

The ERα gene spans eight exons. The coding region is comprised of exons 1-8 where the DBD is encoded by exons 2-3 and the LBD by exons 4-8 (reviewed in Hirata, S. *et al.* 2003). Variants with any of these individual exons or a combination deleted have been found as well as an additional exon S between exons 3 and 4 (Hirata, S. *et al.* 2002). One of the most frequently observed variants in breast cancer is an ERα exon 7 deletion (Δ7) (McGuire, W.L. *et al.* 1991). As with most variants, the Δ7 variant is co-expressed with the wild-type ERα mRNA. It makes up about 30% of total ERα mRNA although only 2 of 23 tumours expressed the protein product of this variant (Fuqua, S.A. *et al.* 1992). Similarly, despite high mRNA levels of this variant in several antioestrogen resistant ER positive MCF-7 cell sublines, a corresponding protein has not been detected (Madsen, M.W. *et al.* 1997). Furthermore, expression differences for the Δ7 mRNA variant could not be revealed between the wild type MCF-7 cells and the different resistant lines suggesting that this variant plays no significant role in the development of antioestrogen resistance. Studies of the predicted proteins from the various mRNA isoforms are rare. There is

still insufficient information as to whether proteins are expressed from the isoforms and whether they fulfil any biological role.

In addition to the coding region, multiple untranslated 5'-exons in the upstream region of exon-1, the promoter region, have been identified (Herynk, M.H. *et al.* 2004). The encoding for an ER mRNA transcript appears to use different promoters employing alternative exons in the 5' untranslated region (UTR). Even though various mRNA isoforms are generated, they only differ in their UTRs. The translation-initiation codon is located downstream from the UTR within the exon-1 region and consequently, all promoters encode the same 66kDa ER protein. The main ERα exon-1 variants identified have been named promoter A-1 to F-1. More recently two new exons have been identified for the ERα promoter called T-1 and T-2 expressed mainly in the testis and epididymis (Brand, H. *et al.* 2002). Isoforms A-F have all been shown to be present in MCF-7 cells as well as benign and malignant breast tissues to varying degrees with A-1 as the most abundant isoform (Flouriot, G. *et al.* 1998; Parl, FF (c) 2000). As with the coding region variants, the function of the 5'UTR variants remains unclear. The alternative exons may be responsible for the control of ER synthesis by regulating the turnover or translation efficiency of the ER mRNA isoforms. This suggestion is based on the thermodynamic principle that the 5'UTR sequences of the mRNA could be folded in the more stable secondary structures (Flouriot, G. *et al.* 1998).

One shorter 46kDa ERα isoform (hERα46) has been isolated in human osteoblasts and the MCF-7 cell line but not in human tissue samples (Flouriot, G. 2000). This mRNA isoform is identical to hERα66 aside from the absence of the first 173aa. It lacks the A/B domain at the N-terminus and consequently the AF-1 region. The hERα46 protein is thought to both homodimerize with itself- and also heterodimerize with hERα66 competing for the ERα DNA binding site. It is able to repress AF-1 dependent transcription indicating a possible mechanism to alter ERα dependent gene transcription.

Similar to ERα, multiple variants have been detected for ERβ. The coding region of the ERβ gene consists of exons 1-7 with several untranslated exons in the upstream region of exon-1 (reviewed in Hirata, S. *et al.* 2003). Like the ERα, ERβ mRNA is a product of a complex transcription initiation using different promoters.

Single as well as multiple exon deletions or duplications have been identified for each of the coding region exons. For example, ERβΔ5, an exon 5 variant lacking part of the LBD has been identified (Inoue, S. *et al.* 2000). This isoform might act as a dominant negative receptor, blocking oestrogen stimulated transactivation by ERα as well as ERβ. An inserted exon M has also been located between exons- 4 and 5 (Shoda, T. *et al.* 2002). It remains to be seen whether any of these isoforms and their possible products give any insight into the function of ERβ, the coexistence and interaction between the two receptor forms and the development of breast cancer.

1.2.4 ER co-regulatory proteins

The oestrogen receptor associates with the promoter of the target gene but also a host of other proteins to mediate transcription. Among those proteins are co-regulatory proteins classified into nuclear receptor (NR) coactivators and NR corepressors, which by definition, enhance and inhibit transcription, respectively.

(i) Coactivators: SRC-1

Among the best-studied coactivators in NR-mediated gene transcription is the p160 protein family with its three closely related members SRC-1 (also termed NcoA-1/ ERAP-160), SRC-2 (also termed TIF2/GRIP1) and SRC-3 (also termed AIB1/ ACTR/ RAC3/TRAM-1). The functionally most interesting similarity in structure is the common central nuclear receptor-interaction domain (NRD) consisting of three LXXLL motifs or NR-boxes, and a common PAS domain (Figure 1.9). The LXXLL motifs account for the coregulator's ability to bind to the LBD of the receptor whereas the PAS domain is known to be involved in protein-protein interaction. Several groups have produced evidence that the p160 family is involved in NR dependent gene transcription. SRC-3 was detected at high levels in the cytosol as well as in the nucleus of MCF-7 cells and has been shown to form an endogenous complex with hER after oestrogen treatment (Tikkanen, M.K. *et al.* 2000). This complex formation also took place to a lesser extent in MCF-7 cells treated with monohydroxytamoxifen, an indication of the involvement of SRC-3 in the agent's partial agonism. However, this experiment failed to detect SRC-1 coimmunoprecipitation with hER. It was suggested that this is partially due to a low level of SRC-1 in the nuclear fractions as opposed to high levels only in cytosolic

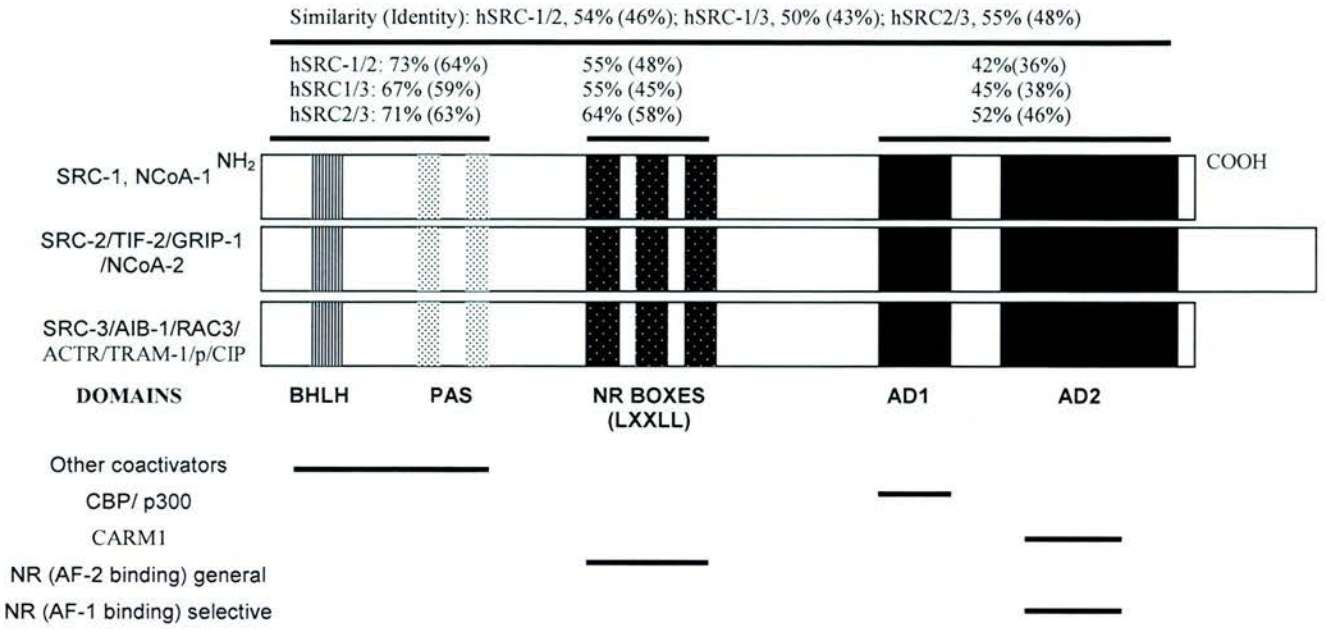


Figure 1.9: Structural and functional features of p160 proteins. The similarity (and identity) of amino acid sequences for full-length human SRC proteins and their specific conserved regions are indicated *above the bars*. The letters below the bars indicate structural domains, and the lines underneath the bars indicate domains that interact with different factors or nuclear receptors (NR) (adapted from Xu, J. and Li, Q. 2003).

fractions. Other groups demonstrated that p160 proteins are essential and sufficient for the initiation of transcription of the E_2 dependent CTSD promoter (Shang, Y. *et al.* 2000). SRC-1 family members were also identified as being recruited to the pS2 promoter in MCF-7 cells as part of an intricate transcription complex assembly leading to gene activation (Métivier, R. *et al.* 2003).

As for all p160 proteins, SRC-1 enhances ERα-mediated gene transcription, but it also mediates full ERα activation by integrating N and C terminal AF-1 and AF-2 functions of the receptor (reviewed in Klinge, C.M. 2000). Specific amino acids in helix 12 within the LBD of ERα have been identified to interact with the conserved LXXLL motif of SRC-1 for cofactor–ER binding. Other coactivators employ different amino acids within the LBD of the receptor indicating the involvement of multiple cofactors at any one time in a transcription complex. SRC-1 has shown to directly interact with other transcription factors such as Fos, Jun, NF-κB and cyclin D1. The activation domains AD1 and AD2, likely to be present in all SRC proteins, recruit CBP/p300 and acetyltransferases, and coactivator-associated arginine methyltransferase (CARM), respectively (reviewed in Nilsson, S. *et al.*

2001). SRC coactivators are thought to primarily accomplish their gene regulatory functions by recruiting such histone modifying enzymatic activities to the receptor. For both, SRC-1 and SRC-2, NR-box 2 has been shown to have the highest affinity for the agonist-bound ERα (Chen, J.D. *et al.* 1995) while NR-box 1 appears to have the highest affinity for SRC-3 (Suen, C.S. *et al.* 1998). SRC-1 is known to have histone acetyltransferase activities stimulating the acetylation of lysine residues on histones H3 and H4 in the receptor chromatin resulting in more extended DNA that is readily available for transcription.

(ii) Coactivators: SRC-2

SRC-1 and SRC-2 show a 54% overall amino acid identity (Xu, J. and Li, Q. 2003). Like SRC-1, SRC-2 has been shown to stimulate E_2 dependent gene transcription. Both activation domains of the receptor, AF-1 and AF-2, are involved in E_2 bound ERα - SRC-2 interaction but only the AF-2 region for ERβ- SRC-2 binding (reviewed in Klinge, C.M. 2000). As with SRC-1, there is an ongoing debate as to whether SRC-2 is involved in not only the oestrogen bound but also the antioestrogen associated receptor gene transcription initiation. Evidence suggests that SRC-1 enhances E_2 as well as 4-OHT stimulated gene transcription by direct association with AF-1 and AF-2 (Smith, C.L. *et al.* 1997; Webb, P. *et al.* 1998). GST pull-down assays have shown that in the presence of SRC-2, the AF-2 region has strong activity when the receptor was bound to E_2 or no ligand while the AF-1 region was activated in the presence of E_2 and tamoxifen (Webb, P. *et al.* 1998). This independent AF-1 tamoxifen initiative has been demonstrated to be due a glutamine rich domain near the C-terminus of SRC-2, a region that is well conserved within p160 proteins, and an extended region of the ERα AF-1 AB domain. However, in MCF-7 cells, SRC-1 did not interact with AF-1 when antioestrogen bound (Halachmi, S. *et al.* 1994). The fact that SRC-2 association with the LBD/AF-2 region might be restricted to the E_2 bound receptor has been reported previously (Cowley, S.M. and Parker, M.G. 1999; Voegel, J.J. *et al.* 1996). Ribbon presentations have been produced to illustrate the structure of the agonist and antagonist associated receptor and its binding to SRC-2. When ERα is bound to E_2 (or another agonist diethylstilbesterol (DES) whose conformation to ERα resembles that of E_2), a peptide derived from the NR box II region of SRC-2 associates with a

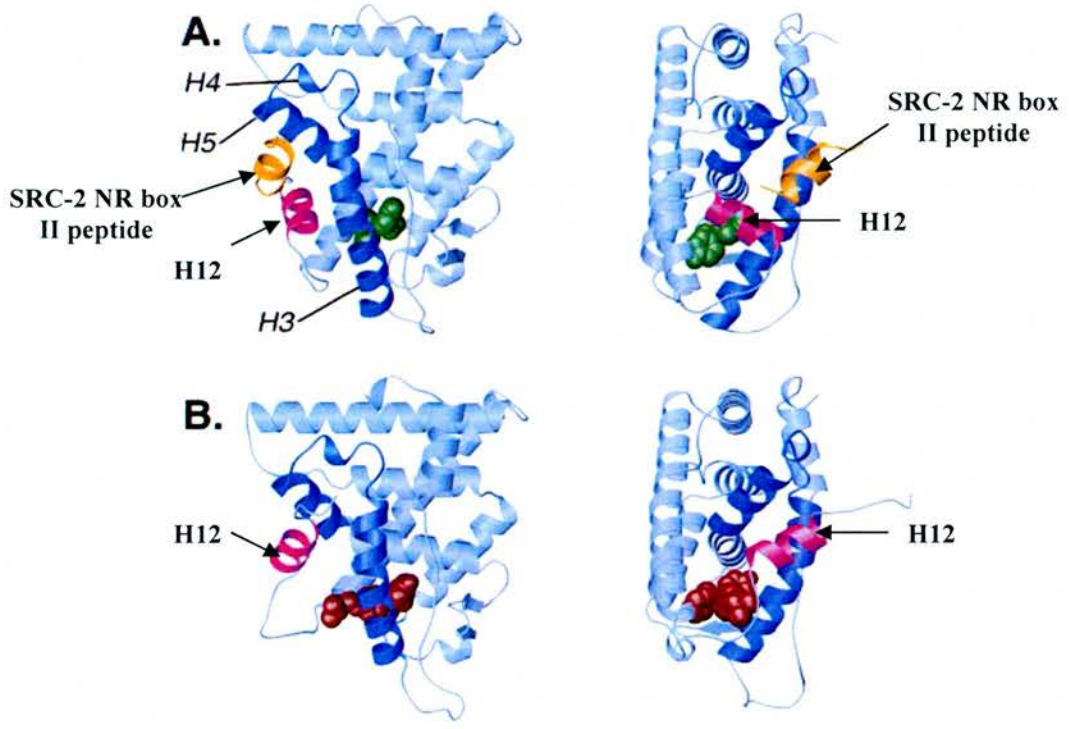


Figure 1.10: Structures of the DES-ERα LBD SRC-2 NR box II complex and of the OHT-ERa LBD complex. **A.** Two orthogonal views of the DES-ERα LBD-SRC-2 NR Box II peptide complex. The coactivator peptide and the LBD are shown as ribbon drawings. The peptide is coloured gold, helix 12 (H12) is coloured magenta, helices 3,4,5 (H3,H4,H5, respectively) are coloured blue. DES is coloured green. **B**: Two orthogonal views of the OHT-ERa LBD complex. The ligand binding domain is pictured as a ribbon. H12 coloured magenta, H3, H4 and H5 blue. OHT is coloured red.

hydrophobic groove on the surface of the LBD (aa at helices 3,4,5,12) (figure 1.10 (A)) (Shiau, A.K. *et al.* 1998). When ERα is 4-OHT occupied, the SRC-2 binding site on the receptor appears to be blocked by helix 12, inhibiting any transcription initiation by interaction of the ERα LBD with SRC-2 (figure 1.10 (B)). These data indicate that SRC-2 does play a role in transcriptional mediation of oestrogen and antioestrogen bound receptor but direct association might be restricted to the AF-1 region of ERα.

(iii) Coactivators: SRC-3

Coactivator SRC-3 has been shown to enhance gene transcription of several nuclear receptors including PR and ERα but not ERβ (Suen, C.S. *et al.* 1998). As mentioned in section 1.2.3 (i), SRC-3 is thought to mediate transcription via interaction of the first of the three binding motifs, NR-box 1, and the AF-2 domain of ERα (reviewed in Nilsson, S. *et al.* 2001). Uniquely, an additional interaction site, a region in the COOH-terminal of the coactivator, has been identified to associate with

the AF-1 domain of the receptor (Webb, P. *et al.* 1998 and others). SRC-3 shows an overall amino acid identity of 50% to SRC-1 and 55% homology to SRC-2. SRC-3 directly interacts with transcription factors pCAF and CBP/p300 and shows intrinsic HAT activity similar to SRC-1. Much research effort has been devoted to SRC-3, also known as AIB-1 (Amplified In Breast cancer 1), since it has been identified as having a prominent role in the development of breast cancer. Overexpression of AIB1 has been detected in *in vitro* and *in vivo* breast cancer. Amplification and high levels of AIB1 expression have been demonstrated in ER-positive MCF-7, BT474 and ZR75-1 cells (Anzick *et al.* 1997). However, elevated expression of AIB1 mRNA in ZR75-1 cells was not confirmed in a different study (Thenot *et al.*1999). T47D and ER-negative MDA-MB-231 cells expressed low levels of the coactivator (Thenot *et al.*1999). In a panel of 105 primary breast tumours, 64% of were found to have a high AIB-1 gene expression relative to expression in normal mammary epithelium and about 10% showed a high AIB-1 mRNA expression by amplification (Anzick *et al.* 1997). Another study demonstrated that the AIB-1 gene was amplified in 4.8% of breast cancers. Its amplification was correlated with ER and PR positivity as well as tumour size (Bautista, S. *et al.* 1998). AIB-1 protein was found to be overexpressed in only 9.8% of breast tumours indicating that high levels of the AIB-1 gene do not necessarily translate into elevated protein levels (List, H.J. *et al.* 2001).

(v) Coactivator and corepressor: RIP140

Other ER interacting cofactors include Receptor Interacting Protein (RIP140) with a molecular weight of 140kDa which interacts with several NRs including ERα in a promoter dependent way. Two regions have shown to interact with the ER, an LXXLL motif at the N-terminal region and a C-terminal domain. RIP140 is a distinct coregulator perhaps of its own category because of its ability to act as a coactivator or corepressor. In MCF-7 cells, RIP140 shows increased binding interaction with the AF-2 domain of ERα in the presence of 17β-oestradiol but not 4-OHT (Cavailles, V. *et al.* 1995). This interaction has been correlated with stimulation of hormone-regulated gene transcription.

Much research on the regulatory role of RIP140 has focused on interactions with NRs other than the ER such as retinoid acid receptors (RAR) or retinoid-X-receptors (RXR) to explain its negative effect on transcription. Nuclear receptor 5A1

(NR5A1/SF-1), a Ftz-F1 member of the NR family involved in endocrine development, steroidogenesis and gonad differentiation, interacts with RIP140 through the AF-2 domain. RIP140 has shown to be a potent corepressor of transcription from a variety of SF-1 responsive promoter genes and acts as an inhibitor of the stimulatory effects of the SRC proteins (Mellgren, G. *et al.* 2003). RIP140 itself is thought to have histone deacetylase (HDAC) activity as one of the means by which it exerts gene repression effects. Unlike classical corepressors, RIP140 interacts with agonist but not antagonist bound receptors. This suggests that this cofactor also does not require antagonist-occupied receptors for its corepressor activities. An example of that is the interaction of RIP 140 with retinoid receptors RAR and RXR, which only takes place with the agonist-occupied dimers (Lee, C.H. and Wei, L.N. 1999).

However, RIP140 might indirectly inhibit several nuclear receptors by ligand-dependent transactivation. RIP140 is thought to compete with other coactivators such as SRC-1 for AF-2, the receptor binding domain, to exert its transcription repression effect. This is illustrated in a study utilizing peroxisome-proliferator-activated receptor (PPAR) responsive reporter gene assays (Treuter, E. *et al.* 1998). The PPAR belongs to a subgroup of NR receptors expressed in MCF-7 and T47D cells (amongst others) and involved in inducing cell differentiation. PPARs bind to DNA bound retinoid X receptors to regulate gene transcription.

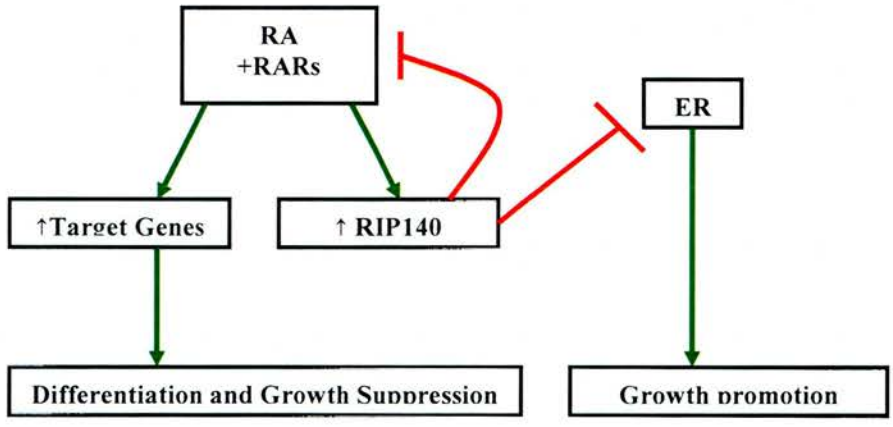


Figure 1.11: Model of RIP140 mediated retinoid acid receptor regulation of gene expression illustrating negative feedback mechnisms (source: Kerley, J.S. *et al.* 2001).

Increasing amounts of RIP140 expression vectors cotransfected with SRC-1 expression vectors showed that RIP140 antagonizes SRC-1 mediated gene transcription. This experiment confirms SRC-1 –RIP140 modulation of PPAR transcription but assigns coactivator functions only to SRC-1 not RIP140. Plasmid transfected RIP140 has been reported to inhibit retinoic acid dependent AR promoter activity in human embryonal carcinoma cells (NT2/D1) (Kerley, J.S. *et al.* 2001). In addition, the RIP140 gene itself is a direct target of retinoid acid being rapidly induced by RA in the same system adding further complexity to RIP140 mediated gene expression. Together with other findings, a model of negative feedback signalling is suggested where RIP140 acts as mediator of cell growth stimulation and suppression (Figure 1.11).

(vi) Corepressors: SMRT and NCoR

Differential recruitment of co-regulatory proteins to the ER transcription complex includes corepressors like Nuclear CoRepressor (NCoR) and Silencing Mediator of Retinoid and Thyroid receptors (SMRT) that have been shown to distinctively interact with the ER in the absence of a ligand and in the presence of an antagonist. This was recently demonstrated in MCF-7 and T47D cells as well as primary breast cancer cell cultures (Fleming, F.J. *et al.* 2004). Beta-oestradiol increased ERα and ERβ –ERE interaction and coregulators SRC-1 and SMRT were identified at the transcription complex. Cells treated with 4-OHT showed increased SMRT expression and ER-ERE binding. Most interestingly, oestradiol treatment lead to preferred SRC-1 binding to the transcription complex whereas with 4-OHT, both oestrogen receptors bound SMRT.

Several isoforms of the 168kDa SMRT protein have been isolated including a longer 270kDa version termed SMRTe or SMRTα (Park, E. -J. *et al.* 1999). Isoforms for the 270kDa NCoR protein include RIP13Δ1 with a deletion in the nuclear receptor interaction domain (ND) 3 and RIP13a with a deletion in the repression domain (RD) 3. A recent comprehensive review published by Privalsky, M.L. (2004) compares structure and functionality of corepressors SMRT and NCoR. The two corepressors show a common molecular structure with a 45% aa identity (Ordentlich, P. *et al.* 1999; Park, E.J. *et al.* 1999). The N-terminal region harbours four and three

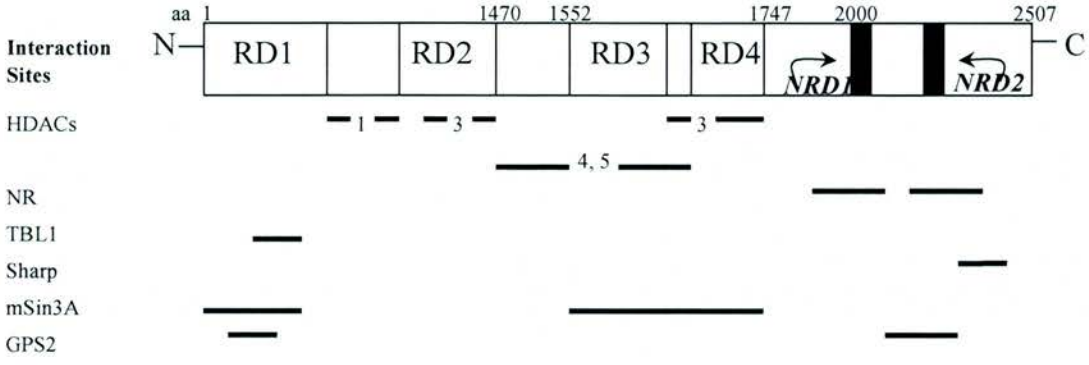


Figure 1.12: Schematic diagram of hSMRTα/e and its structural and functional features. The locations of the nuclear receptor interaction domains (NRDs), repression domains (RDs) and the interaction sites of other possible components of the transcription corepressor complex are shown below. Histone DeACetylases=HDACs; Nuclear Receptor=NR; Transducin-Like protein=TBL1; mammalian Switch independent 3 protein=mSin3A; G Protein Pathway Suppressor 2=GPS2 (source: adapted from Privalsky, M.L. 2004 and Klinge, C.M. 2000).

repression domains for SMRT and NCoR, respectively, while the C-terminal includes two and three nuclear interaction domains (figure 1.12). Similar to NRD functions in the structure of p160 coactivators, NRDs in SMRT and NCoR are responsible for the interaction of the corepressor directly with the receptor. NRDs contain a L/IXXI/VI sequence embodied in a motif named CoRNR box comparable to coactivator NR-boxes (Hu, X. and Lazar, M. 1999). CoRNRs are the sites that build contact between the corepressor and specifically structured helices 3.5. and 6 at the receptor (reviewed in Dobrzycka *et al.* 2003). As mentioned, the NR docking site at the receptor is thought to be accessible only in the non-liganded or antagonist bound state. NRs including ER, PR and AR bind SMRT and NCoR with binding affinities of varying degree. Binding of the corepressor to the receptor gives a distinct conformation leading to a cascade of events and to recruitment of other transcription factors at the repression domains. NCoR and SMRT have shown to interact with numerous proteins such as HDACs 3/4/5 and 7 to deaccess DNA for transcription (Guenther, M.G. *et al.* 2001; Yu, J. *et al.* 2003). Both corepressors have been identified as part of large transcription complexes to regulate transcription (Shang, Y. *et al.* 2000; Fischle, W. *et al.* 2002). Transducin-like protein1 (TBL-1) may assist to recognize chromatin substrates and stabilize the quaternary complex structure. G-protein pathway suppressor 2 (GPS2) is thought to bind to TBL-1 and NCoR and further mediate transcription complex assembly (Privalsky, M.L. 2004).

The mechanism of cofactor recruitment and transcription complex assembly is discussed in more detail in subsequent chapters.

As a result of the multiplicity of transcription factors recruited by corepressors, SMRT and NCoR are often referred to as 'platform proteins' acting as the base for many different proteins to display their functions. Corepressors are then able to exert their function by different mechanisms utilizing functions of the docking proteins, such as chromatin remodelling functions or effects on the basal transcription apparatus. In addition, corepressors directly compete for NR binding sites, similar to coactivators.

(vii) Other corepressors

There is a growing list of additional corepressors such as Repressor of Estrogen receptor activity (REA) and BRCA1, a protein product of the breast cancer susceptibility gene, LCoR and MTA1/1s but they are less well studied (reviewed in Privalsky, M.L. 2004). Semiquantitative PCR analysis showed varied expression of REA mRNA in normal and neoplastic breast tissue (Dotzlaw, H. *et al.* 1999). Moreover, expression might positively correlate with estrogen receptor levels and inversely with tumor grade (Simon, S.L.R. *et al.* 2000). The REA gene encodes a small 37kDA protein. It interacts with the E_2 or 4-OHT bound ER receptor through direct association with the ER –LBD as well as indirectly through binding to other transcriptional factors to repress receptor activity (Delage-Mourroux, R. *et al.* 2000). Similar to RIP-140, REA interacts with SRC-1 actively competing with the coactivator preventing it from exerting its stimulatory effect on transcription initiation. Supporting this mechanism, an LXXXLL motif within REA has been identified to associate with SRC-1 but not the oestrogen receptor. REA enhances the antagonistic activities of antioestrogens such as 4-OHT-bound to ER but also suppresses the agonist activities of oestrogen bound to the receptor (Montano, M.M. *et al.* 1999). Furthermore, suppression of hormone liganded-ER mediated transcription could be due to REA association with PTα, a chromatin remodelling protein (Martini, P.G.V. and Katzenellenbogen, B.S. 2003). The direct and indirect binding to other cofactors or the receptor enables REA to modulate hormone and antihormone mediated transcription in a cofactor and ligand specific manner in breast and other cancer cells.

1.3 Oestrogen action

1.3.2 ER mediated genes

The number of identified ER regulated genes is constantly increasing. The complexity of E_2 action is reflected in a multi gene expression profile and their tissue specific, ligand and ligand-dose dependent expression. Investigations mostly using microarray analysis have been carried out to identify genes which are overexpressed in breast cancer cell models and primary cancer cells and can be associated with disease outcome or prognosis and classifications of primary tumours. The large scale investigation of ER linked genes is demonstrated in a global approach using microarrays containing probes representing approximately 19.000 human genes (Lin, C.Y. *et al.* 2004). The study identified 386 oestrogen sensitive genes in T47D cells of which 137 were ER –regulated when treated with either E_2 or E_2 in combination with ICI 182,780 or protein synthesis inhibitor cycloheximide; 89 of which were proposed to be direct target genes as opposed to downstream targets. ER- regulated genes were defined by being responsive to oestrogen but blocked by ICI 182,780. The direct target genes were further categorized as being 59 E_2 up-regulated and 30 E_2 down-regulated genes. The list contains targets such as cell signalling proteins adenylate cyclase 1 (ADCY2), transcription regulation proteins such as nuclear receptor interacting protein 1 (NRIP1), cell cycling proteins such as CTSD and multiple proteins with unknown functions.

In a different study, the effect of the antioestrogens raloxifene, *trans*-hydroxytamoxifen (4-OHT) and ICI 182,780 on 40 E_2 up- and 89 down-regulated genes in MCF-7 cells, revealed fundamentally different expression profiles indicating distinct biological mechanisms for each agent (Frasor, J. *et al.* 2004). For both, E_2 up- and down-regulated genes, ICI 182,780 produced the most potent antagonistic effect on 95% and 91% of genes, respectively. Raloxifene and 4-OHT also act as antagonists of E_2 up-regulated genes (67% and 47%), but act as partial agonist/ antagonists in the majority of the down-regulated genes, 63% and 43%, respectively. Most agonistic activity was found for 4-OHT in 23% of the up- as well as 31% of the down-regulated genes. This study identified single oestrogen regulated

genes like the transcription factor c-fos or DNA synthesis regulator CDC6 showing unique expression patterns in response to ICI, raloxifene and 4-OHT and demonstrates that E_2 mediated transcription is part of a vast number of pathways. Several oestrogen –regulated genes have been extensively studied and those include the progesterone receptor, pS2 and cathepsin D.

(i) Progesterone Receptor

Also a steroid hormone, progesterone mediates most of its known effects by binding to a further member of the nuclear receptor family, the progesterone receptor (PR) whose expression and regulation is highly influenced by oestrogen. Progesterone action has been shown to play a key role during normal female reproduction. This includes functions in the uterus and the ovary with the control of ovulation and maintenance of pregnancy; proliferation and differentiation, control of lactation in the mammary gland; or the brain where progesterone mediates signal transduction required for the expression of sexual responsiveness (Graham, J.D. and Clarke, C.L 1997). The intricate mechanisms of progesterone action and its complex interaction with other steroid receptor pathways has been demonstrated in phenotypic studies of the progesterone knockout mouse model (PRKO) (Lydon, J.P *et al.* 1995). Severe physical reproductive abnormalities and impaired sexual behavior were exhibited as a result of the absence of the PR gene in these organisms.

The two progesterone receptor isoforms hPR-A and hPR-B have been identified as functionally different receptors. The two proteins show 100% aa homology with the exception of an additional 164 aa at the N-terminal end of hPR-B (Clarke, C. and Sutherland, R.L. 1990). Both isoforms are generated from transcripts from a single gene but encoded by separate oestrogen responsive promoters resulting in either the 94kDa hPR-A protein or a 115 kDa hPR-B protein. (Giangrande, P.H. and McDonnell, D.P. 1999). Structural and functional features of both progesterone receptor isoforms resemble other nuclear receptor family members (Figure 1.13) (Mulac-Jericevic-B. and Conneely, O.M. 2004). Within the six main functional domains, the A/B region plays a key role in mediating gene activation of target genes by recruiting coactivators and corepressors in a ligand independent manner to the transactivation domain AF-1. Unique to hPR-B, its extended N-terminal region

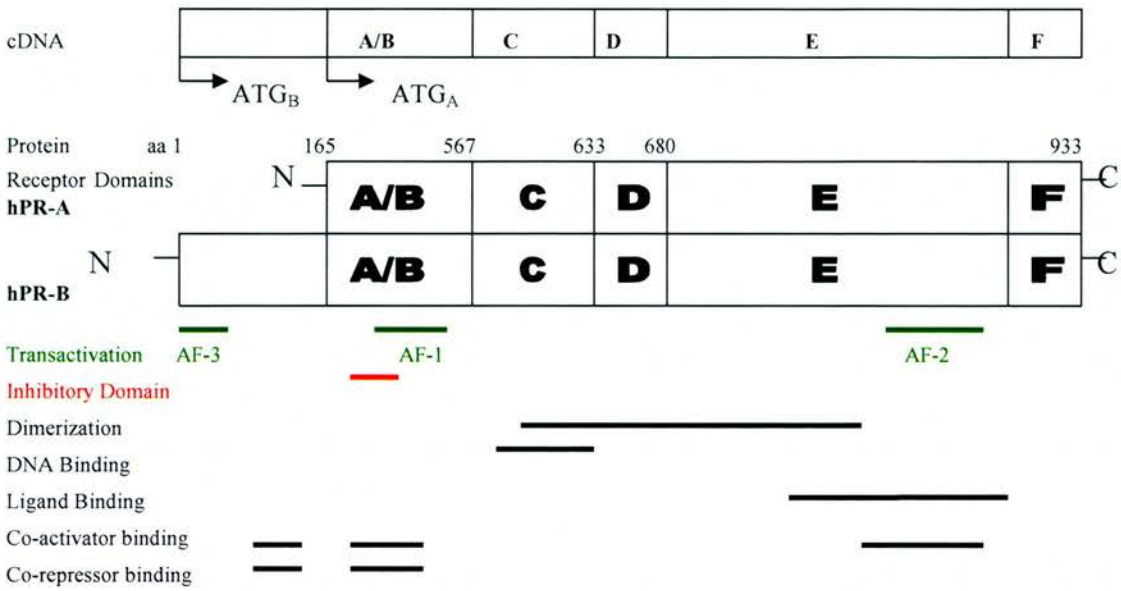


Figure 1.13: Schematic diagram of progesterone receptor protein and its structural and functional domains. Numbers note amino acid positions within each protein (adapted from Graham, J.D. 1997 and Mulac-Jericevic, B. 2004).

contains an additional transactivation domain, AF-3 (Sartorius, C.A. *et al.* 1994; Huse, B. *et al.* 1998). This region might be responsible for the stronger activation potential of hPR-B, augmenting activity of hPR-B and preventing inhibitory functions of hPR-A on itself (Graham, J.D. and Clarke, C.L 1997). The inhibitory function of hPR-A is the result of an inhibitory domain (ID) within the A/B region of progesterone receptors. Both receptors contain this region but it is only active in the hPR-A isoform where it can act as a repressor of hPR-B and possibly other receptors (Huse, B. *et al.* 1998). The inhibitory domain has also been identified to serve as a docking site for transcriptional corepressors (Giangrande, P.H. *et al.* 1997). The ligand binding domain, located within the E-region of the protein towards the carboxyl-end, is responsible for hormone binding but also contains a transactivation domain, AF-2, specifically associating with coactivators in a hormone dependent manner. The DNA binding domain within region C enables the receptor to directly bind to its specific target genes.

Known direct or indirect progesterone target genes include growth factors and growth factor receptors such as EGF, TGFα/β and IGF or cell cycle genes such

as cyclin D1 and c-*myc* (Mulac-Jericevic-B. and Conneely, O.M. 2004). Although both PR isoforms bind progestins, their transactivation functions are different and are promoter as well as cell specific. While hPR-B is thought to primarily activate transcription of target genes, hPR-A has shown to transcriptionally repress progesterone target genes. Moreover, hPR-A can inhibit the stimulatory actions of hPR-B if coexpressed indicating differential cofactor binding of both isoforms (Giangrande, P.H. and McDonnell, D.P. 1999). This allows for transrepression of other pathways such as that of the oestrogen or the androgen receptor. Corepressor SMRT has been demonstrated to preferrentially interact with the inhibitory domain of hPR-A over hPR-B. When PR is antagonist bound, hPR-A becomes inactivated while hPR-B becomes a strong transcriptional activator.

Whilst progesterone decreases, oestrogen increases PR expression. In other words, progestins oppose E_2 mediated action, which is thought to be the basis for the main effects of PR. This is thought to be due to a downregulation in ER transcription as decreased ER protein but also mRNA have been found in response to progesterone treatment in breast cancer cells (Graham, J.D. and Clarke, C.L 1997). During normal mammary gland development, progesterone plays particularly vital roles during two specific developmental phases, puberty and pregnancy. Cells expressing the progesterone receptor segregate from proliferating cells. Normal breast development shows characteristic changes in PR expression pattern from an undifferentiated, even expression in puberty to an epithelial clustered concentration in the adult. During pregnancy, progesterone exposure has an increased proliferation effect on ductal epithelium and differentiation effect in alveolar lobules. This response also results in activation of growth factors of PR negative cells. However, in breast cancer the distinctive separation of PR containing cells from normal proliferative cells no longer takes place (Graham, J.D *et al.* 1996). Some research effort has been made to identify the role of the PR isoforms in breast cancer with inconclusive results. While the isoform expression ratio appears approximately even in non-malignant tissue, ratios seem to vary to a great extent in tumours and might be linked to tumour stage and invasiveness (Graham, J.D *et al.* 1996 and Mote, P.A. *et al.* 2002). Predominant expression of one form or the other might indicate alternative pathways in endocrine response and potential antihormone resistance mechanisms.

This has sparked an ongoing discussion as to whether PR testing has its place in the modern breast cancer diagnostic regime as a therapeutic indicator. A recent correspondence between two research groups published in *The Journal of Clinical Oncology* outlines the main arguments. It is reasoned that testing has been used to identify a relatively small group of about 3-5% of patients with ER- and PR+ tumours who might benefit from endocrine therapy (Olivotto, I.A *et al.* 2004). Further, in ER+ patients, PR testing is not necessary given that most ER+ patients receive endocrine therapy regardless of their PR status. However, PR testing can identify a subgroup of ER+ tumours, ER+/PR-, which are less likely than ER+/PR+ groups to respond well to hormone therapies (Fuqua, S.A. *et al.* 2005). In addition, it is argued that studies have recognized a generally poor response to adjuvant tamoxifen in the ER+/PR- patients. This subgroup of about 30% might therefore benefit from testing. In the light of more recent therapy options, preliminary studies also indicate potential differences in response when comparing tamoxifen treatment

Clinical evidence	**Scientific evidence**
ER- tumours ER-/PR+ tumours respond better to endocrine therapy (Robertson, J.F. et al. 1996)	ER- tumours ER-/PR+, no evidence of intact ER signalling
ER+ tumours ER+/PR- benefit less from hormonal therapy, part.tamoxifen (Osborne, C.K. et al. 1980) ER+/PR- benefit from anastrazole more than tamoxifen (ATAC trial: Baum, M. et al. 2002; Dowsett, M. 2003) ER+/PR+ might benefit from tamoxifen therapy followed by anastrazole ER+/PR- might benefit from anastrazole followed by tamoxifen	ER+ tumours In ER+/PR- tumours, evidence of altered ER signalling and enhanced growth factor signalling (Cui, X. et al. 2003)
PR+ tumours hPR-A rich tumours might benefit less from tamoxifen therapy (Hopp, T.A. et al. 2004)	PR+ tumours hPR-A rich tumours show increased aggressiveness (Mote, P.A. et al. 2004)

Table 1.4: Scientific and clinical issues in the controversy surrounding the usefulness of progesterone testing in breast cancer treatment as argued by Olivotto, I.A *et al.* 2004 and Fuqua, S.A.W. *et al.* 2005.

with anastrazole treatment for ER+/PR+ and ER+/PR-tumors. And lastly, it is argued that based on a pilot study, tumours expressing the PR isoform hPR-A in abundance might benefit less from tamoxifen therapy (Fuqua, S.A.W. *et al.* 2005). PR testing would therefore identify a subgroup of PR expressing tumours for whom endocrine therapy is not the preferred treatment. The key issues of this debate are highlighted in table 1.4 comparing clinical and scientific arguments. The role of the progesterone receptor in the development of breast cancer remains unclear. Known complex interaction between hormone receptor pathways, newly identified receptor isoforms and their differential functions indicates enormous potential in alternative endocrine signalling during malignant breast development. Whether progesterone testing should become obsolete may have to be re-evaluated as research progresses.

(ii) Trefoil factor 1/ pS2

The function of trefoil factor family (TFF) proteins in breast cancer remains unknown. As TFF proteins are predominatly expressed in the mucus in the gastrointestinal tract (GI), they are thought to be involved in the maintenance and organization of the mucous layer lining. They have also been identified, in several but not all, normal and malignant breast epithelial cell lines and tumours at varying levels of expression (May, F.E.B. and Westley, B.R. 1997; May, F.E.B. *et al.* 2004). It has been suggested that motogenic characteristics of TFFs detected in the repair of gastric tissue could also play a role in directing the invasion and expansion of breast cancer cells. The original observation was made in tissue surrounding gastric ulcers in rat models (Taupin, D.R. *et al.* 1994). A strong increase in trefoil factor expression led to the proposition that the migration of cells over the damaged surface was mediated by such peptides. A recent study has indeed shown that TFF2 could stimulate breast cancer cell movement (May, F.E.B. *et al.* 2004).

Three human trefoil factor proteins have been identified: pS2 (TFF1/ pNR-2/ BCEI), the first and best studied 60-residue protein; TFF2 (spasmolytic peptide - hSP) a 106-residue protein; and TFF3 (intestinal trefoil factor -hITF), another small of 60-residue protein (reviewed in Ribieras, S. *et al.* 1998). A small 42-43 residue 'trefoil sequence'characterizes the family. pS2 and TFF3 contain a single trefoil domain whereas TFF2 contains two such sequences.The human TFF genes are

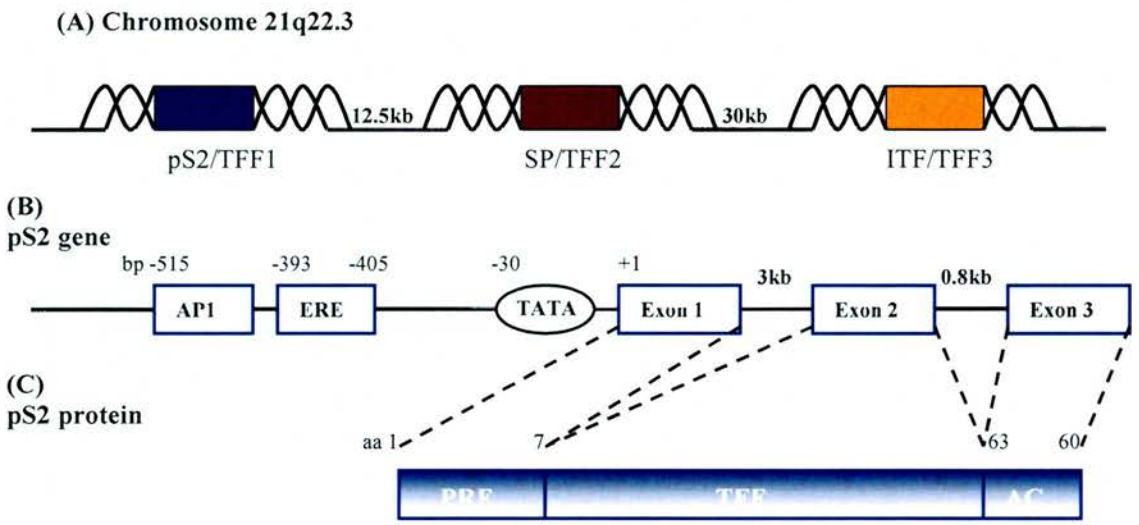


Figure 1.14: (A) TFF gene cluster at on human chromosome 21q22.3, kb =kilobases. (B) pS2/TFF1 gene structure: bp= base pairs; AP1= jun/fos responsive element; ERE= Estrogen response Element; TATA- TATA box. (C) pS2/TFF1 protein structure: aa= amino acids; PRE= signal peptide domain; TFF domain and AC= carboxy-terminal acidic domain (adapted from Ribieras, S. *et al.* 1998).

located in close proximity on chromosome 21q22.3 spanning a genomic DNA fragment of 55kb. pS2 is positioned 12.5Kb upstream TFF2, which is found 30Kb upstream of TFF3 (Figure 1.14 (A)).

The promoter of the 4.5kb pS2 gene contains an imperfect 13-base pair oestrogen response element between positions –405 and –393, a jun/fos responsive AP-1 site as well as DNA enhancer elements responsive to the epidermal growth factor, the tumor promoter TPA or the c-Ha-ras oncoprotein (Berry, M. *et al.* 1989; Nunez, A.M. *et al.* 1989) (Figure 1.14 (B)). The small 6.5kDa pS2 protein is encoded by three exons translating into the amino-terminal signal peptide (exon 1), the TFF domain (exon 2) and the carboxy –terminal acidic motif (exon 3) (Figure 1.13 (C)). The highly conserved pS2 trefoil sequence includes six cysteine residues that form three intramolecular disulphide bonds resulting in three loops within the TFF sequence (May, FEB *et al.* 2000 and Polshakov, V.I. *et al.* 1997) (Figure 1.15). A seventh cysteine residue near the C-terminus can form intermolecular disulphide bonds (reviewed in May, F.E.B *et al.* 1997). The loops are stacked into the characteristic cloverleaf -like disulphide structures. This compact structure is stable and resistant to proteases and thiol agents. Structural analysis has suggested that

(A) Human TFF1/pS2 amino acid sequence

(B) Human TFF1/pS2 2D structure

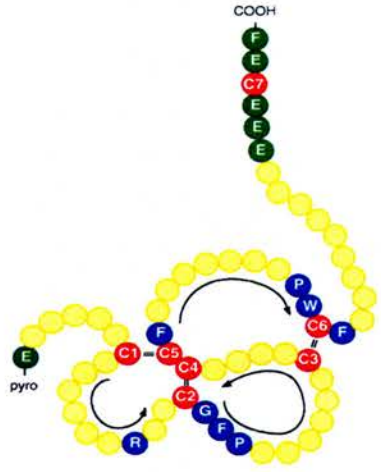


Figure 1.15: (A) Amino acid sequence of TFF1/pS2 protein. Cysteine residues involved in disulphide bonding are indicated in red. (B) TFF1/pS2 2D protein structure. Red and blue residues indicate conserved aa of trefoil domain. Three disulphide bonds are shown (=). C7 and green aa represent Ac domain. (Figure (A) Polshakov, V.I. *et al.* 1997 and Figure (B) Ribieras, S. *et al.* 1998).

exposed surface residues could serve as binding sites for receptors and ligands (Polshakov, V.I. *et al.* 1997).

pS2 is highly expressed in ER positive breast cancer cell lines and malignant breast cancers. Low-level pS2 mRNA and protein have also been detected in normal mammary tissue (Predine, J. *et al.* 1992 and others). A study involving 172 primary breast cancers found that 68% of the tumours expressed pS2 protein (Henry, J.A. *et al.* 1991). It has been firmly established that pS2 mRNA and protein expression is induced by oestrogen (Masiakowski, P. *et al.* 1982 and May, F.E.B and Westley, B.R. 1986). The hormone induction of pS2 is thought to be a primary response to oestrogen and based on the ER binding to the ERE within the promoter of the sequence (Berry, M. *et al.* 1989). Antioestrogens reverse the effect of oestrogens on

pS2 expression. Non-steroidal antioestrogens act as partial oestrogen agonists (May, F.E.B 1987and Westley, B.R. 1987). In addition, pS2 mRNA levels have been positively associated with the presence of ER and PR (Henry, J.A. *et al.* 1991 and others) and inversely associated with histological grade and size of some tumour forms (Predine, J. *et al.* 1992). There is evidence that a high pS2 expression is generally associated with a good prognosis. This observation might be based on the association of pS2 expression with ER/PR expression where tumours have better response rates to anti-hormonal treatment and hence a better prognosis. The expression of pS2 has shown to be an indicative tool for therapy success in neoadjuvant hormonal therapy for postmenopausal breast carcinomas (Soubeyran, I. *et al.* 1996 and others). Consistent correlation remains absent for the association of pS2 status and other breast cancer factors such as cell type, patient age, lymph node status or overall survival time. Consequently, the prognostic value of pS2 expression with regards to disease progression and therapy response is limited.

(iii) Cathepsin D

Similar to pS2, Cathepsin D (CTSD), a lysosomal protease, is a potential prognostic factor for breast cancer when not only the presence in MCF-7 and ZR75-1 breast cancer cell lines was established but its oestrogen inducibility confirmed in ER+ cells (Rochefort, H. *et al.* 1987). It has since been confirmed that high or moderate levels of CTSD expression in primary tumours correlate with an increased risk of relapse and overall survival independent of histological grade, ER status or tumour size (Foekens, J.A. *et al.* 1999). proCTSD is a 56kDa precursor of the CTSD protein that associates with the mannose 6-phosphate/ insulin like growth factor-II receptor (M6P/ IGF2R) (detailed review by Rochefort, H. *et al.* 2000). Release of the protein from its receptor leads to the synthesis of a 48kDa intermediate and a mature form of CTSD consisting of a 30 kDa and a 12 kDA protein. It is the mature form that has been shown to play a role in breast cancer metastasis.

As a protease, cathepsin D degrades proteins but has also been shown to play a role in the development of newborns in protection from intestinal necrosis and thymic apoptosis. In breast cancer, it has been suggested that CTSD has motogenic characteristics facilitating the invasion and spread of cancer cells. Abundant proteases in tumour cells are released and mediate extracellular matrix digestion in

surrounding tissue to allow cancer cells to invade connective tissue or the blood stream. Thus, there is an ongoing controversy as to whether it is the intracellular levels of CTSD stored in lysosomes and phagosomes, or the extracellular levels secreted by the cells that are of prognostic value. It has been shown that breast cancer cells produce high levels of CTSD, which is not stored but released into extracellular space (Capony, F. *et al.* 1989). A recent study compared the effect of oestrogen and tamoxifen on intracellular and extracellular CTSD activity (Dabrosin, C. *et al.* 2004). Oestrogen was shown to stimulate intracellular proteolytic activities and secretion of CTSD, whereas tamoxifen stimulated intracellular levels but decreased CTSD secretion. Opposing effects of both ligands were observed for the M6P receptor. Receptor levels were decreased with oestrogen exposure and increased with tamoxifen indicating that the secretion of CTSD might be M6P receptor regulated. The authors conclude that it might be vital to measure CTSD levels at different sites to determine its biological role and usefulness as prognostic marker.

1.3.3 ER –ERE interaction

Traditionally, ER mediated gene transcription was thought to be based on a direct contact between the oestrogen-bound ER and the oestrogen response element (ERE) within the promoter. However, experiments have shown that ligand binding and direct ER-ERE interaction present just small elements of transcriptional initiation and are part of a much more complex mechanism.

The ERE, a 13bp palindrome located mainly within the promoter of an oestrogen responsive gene, serves as DNA binding site for the receptor (reviewed in Klinge, C.M. 2001). The ERE consensus 5'GGTCA nnn TGACC 3', where n serves any nucleotide, can confer oestrogen responsiveness to a reporter gene when transfected into cell lines (Klein-Hitpass, L. *et al.* 1988). A specific additional nucleotide on either side of the palindrome extends the ERE to a 15bp palindrome (5'AGGTCA nnn TGACCT 3'). The number, spacing and nucleotide sequences immediately adjacent to the ERE vary frequently between genes and can increase or decrease the ER binding affinity (Klinge, C.M. *et al.* 1992; Driscoll, M.D. *et al.* 1998). The ER is able to adapt competently and bind to individual nucleotide changes and resulting asymmetries. The receptor binds to one half side of the single or double stranded

ERE with its conserved element and tolerates variability in the other non-corresponding half sequences (Parl, F.F. (c) 2000). Experiments have shown that the binding affinity and transcriptional activity is inversely proportional to the number of nucleotide changes from the consensus within one of the ERE halfsites (reviewed in Klinge, C.M. 2001). It remains controversial as to whether the ER binds simply as a homodimer to the halfsites of the ERE, or also as a heterodimer. The ER might bind as a heterodimer when bound to coregulatory proteins while the two flanking dinucleotides on both sides stabilize the complex. The extended 5' half site of the ERE is also recognized by other receptors such as RAR, Thyroid Receptor (TR) or vitamin D3 receptor (VDR). Orientation and spacing of the sequence is what assures specificity of receptor- response element binding.

The ER exerts its gene regulatory function through direct association with the ERE activated by oestrogen, through complex formation with other regulatory

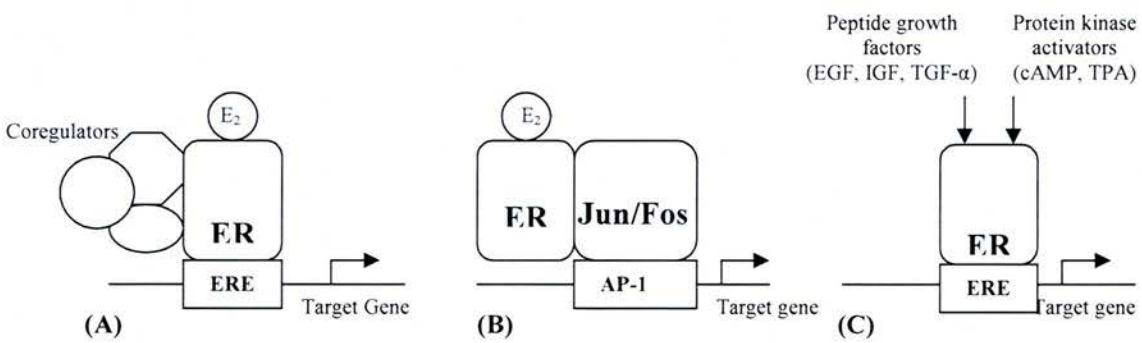


Figure 1.16: Simplified schematic representation of different pathways of ER-mediated gene transcription. (A) Classical pathway involving direct E_2-bound ER –ERE interaction. After E_2 binding, the receptor forms homo- or heterodimers and associates with the ERE of the target gene. (B) Non-classical ERE –independent pathway involving the interaction of the E_2 bound ER with other transcription factors such as Jun and Fos to their response elements (AP-1). (C) E_2 independent transcription involving the ER activation by signalling from extracellular growth factors such as IGF or protein kinase activators such as cAMP (adapted from Parl, F.F. (c) 2000).

factors one of which will directly associate with its response element (RE), or direct association with the ERE but activated by a ligand other than oestrogen (see figure 1.16). The indirect or steroid-independent mechanism involves activation through peptide growth factors such as epidermal growth factor (EGF) or transforming growth factor α (TGFα) via tyrosine kinase receptors; and protein kinase activators A and C such as cyclic AMP (cAMP)(Figure 1.16 (C)). There is extensive evidence to suggest crosstalk between the oestrogen and growth factor pathways, and the ER's

ability to be activated by phosphorylation (Lannigan, D. 2003). Phosphorylation of the receptopr by growth factors can activate the oestrogen receptor (Hermanson, O. *et al.* 2002). The combination of oestrogen and growth factors can mediate gene transcription synergistically.

ER signalling by association with another transcription factor bound to its response element, also called 'tethering', has been demonstrated with the ubiquitous transcription factors Sp1 or AP-1 (Figure 1.16 (B)). The response element for Sp1, Sp1RE, is found close to EREs in multiple genes such as CTSD, c-myc or RARα (reviewed in (Parl, F.F. (c) 2000). Two AP- pathways have been proposed (Webb, P. *et al.* 1995 and Kushner, P.J. *et al.* 2000). ERα with oestrogen or tamoxifen utilizes the AF pathway to activate AP-1 by association with AP-1 ligands Jun and Fos and the recruitment of additional cofactors. Proteins such as the p160 family and CBP/p300 might serve as a link between Jun/Fos and AF-1/ AF-2 domains of the receptor leaving the ER –DBD dissociated and unoccupied from the ERE. ERβ and AF-1 deleted ERα activate AP-1 in an AF independent manner in the presence of antioestrogens raloxifene and ICI 182,780. Here, the receptor binds nuclear corepressors as mediators and the ER –DBD is directly involved.

1.3.4 Mechanism of ER -ERE action

The functions of the ER mediated network are determined by the cellular context. The specific action of the ER depends on the subtype and isoform of the receptor itself, the ligand, the target gene and more specific, the gene regulatory DNA site (ERE) and the availability and characteristics of a host of coregulator proteins. Recent evidence suggest a dynamic process where large transcription complexes are assembled to initiate or repress gene transcription in an ordered and combinatorial manner.

(i) Assembly of ER transcription complexes to mediate gene transcription

There are a number of approaches to describe the molecular actions of the E_2 dependent ER activation. The assembly of different transcription complexes in a sequential, combinatorial or parallel manner forms the basis for most mechanisms (detailed review by Glass, C.K. and Rosenfeld, M.G. 2000; and Hermanson, O. *et al.* 2002). Members of the complexes initiate transcription by reorganizing the

chromatin structure and/ or modifying and recruiting additional transcription factors, basal transcription factors and RNA polymerase II (figure 1.16). Two forms of chromatin remodelling complexes are thought to be crucial. The ATP dependent BRG (SWI/SNF) complex reorganizes local nucleosome structure and mediates assembly of further complexes by attracting sequence specific transcription factors to the DNA. Complexes involving P/CAF, CBP/p300 and p160 protein possess histone acetyl transferase (HAT) activities. Transcriptionally active chromatin fractions are enriched in acetylated histones. This altered nucleosome structure permits access for the RNA polymerase II (pol II) machinery initiating transcription. The level of acetylation of the genome is then linked to the level of transcription. The P/CAF complex might be composed of numerous proteins; amongst them so called SAGA/ ADA proteins and a group of TATA -binding -protein (TBP) related proteins called Spt proteins. Coactivators CBP/p300 are involved in many signalling pathways. They show HAT activities and facilitate other protein complexes. Despite their ER binding sites, it is unknown whether they directly associate with the receptor. p160 family proteins might primarily serve as platform proteins via their highly conserved PAS domains. Protein-protein interactions have been demonstrated with other transcription factors such as CBP or CARM1. As mentioned earlier, SRC1 and SRC-3 also show HAT activities. In addition, a large coactivator complex, the TRAP/DRIP/ARC complex, might directly connect the ER to the basal transcription machinery containing RNA polymerase II (Pol II) with basal transcription factors (Glass, C.K. and Rosenfeld, M.G. 2000).

Corepressors form multiunit complexes opposing the effects of coactivators. NCoR and SMRT are thereby thought to act predominantly as recruitment and bridging proteins attracting factors with histone deacetylase (HDAC) functions as both corepressors lack intrinsic deacetylation domains (Klinge, C.M. 2000). HDACs maintain chromatin in a condensed inaccessible state. Factors found to be associated with NCoR and SMRT include mSin3, HDAC 1/2/3/4/5/7, BRG-1 transducin β-like protein 1 (TBL-1). Repressive functions are thought to be a result of corepressors mediating NRs in directly repressing target genes or by antagonizing coactivators thereby indirectly inhibiting transcription. During active repression corepressor

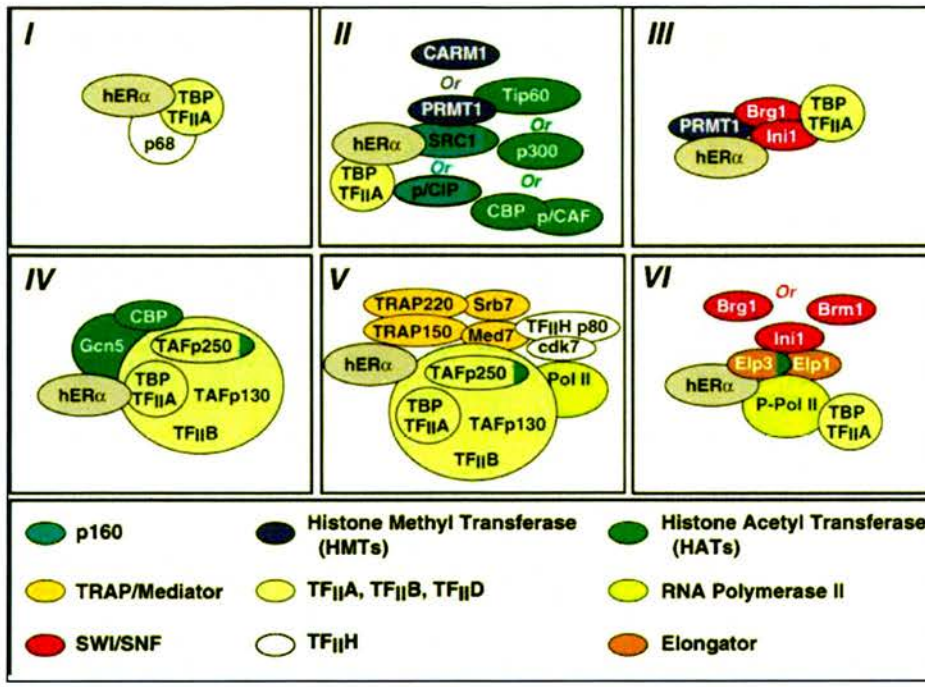


Figure 1.17: pS2 gene activation: Proteins assembled in six different transcription complexes identified at the pS2 promoter. (source: Métivier, R. *et al.* 2003).

complexes are recruited in the presence or absence of ligands, bind to the ER -ERE and facilitate chromatin condensation. Indirect repression, also called transrepression, involves activation of the receptor, which induces the upregulation of corepressors. Corepressors have also been shown to compete actively with coactivators to alleviate transcriptional activation. Overexpression of coactivator proteins CBP/p300 has been shown to reduce transrepression by NR (Glass, C. and Rosenfeld, M.G. 2000). As with coactivators, distinct complex compositions incorporating one or more of these factors have been identified and suggest corepressor associations are promoter and cell-type specific.

Promoter specific protein complexes have been demonstrated in a recent study examining transcriptional activation at the pS2 promoter (Métivier, R. *et al.* 2003). Six different combinations of cofactors have been identified after re-analysis of samples initially used to identify cofactors via chromatin immunoprecipitation analysis (ChIP) (see figure 1.17). The ERα is required in all complexes but other factors are not always part of the complex. For example, complex II contains HATs and HMTs crucial for chromatin modulation making the promoter available for transcription. Protein complex VI containing RNA pol II or DNA elongators Elp1

and Elp2 appears to be part of the actual transcriptional initiation where chromatin remodeling enzymes are no longer needed. These results suggest a certain functional specificity for each cofactor and underline the hypothesis that an alternate and combinatorial assembly of transcription complexes determines the transcriptional output.

(ii) Dynamics of ER mediated transcription

The identification of this large number of cofactors involved in ER-mediated gene transcription led to studies analyzing the dynamics of events leading to gene activation. One of the first groups to describe the kinetics of the transcription complex assembly based on ChIP experiments was that of Shang,Y. *et al.* (2000). The oestrogen receptor was found to bind to the promoter and the sequential recruitment of p160 coactivators, CBP, p300, pCAF, and PBP shown after oestrogen binding. It was demonstrated that the target promoter is continuously involved in complex binding and dissociation providing evidence for a cyclic model of transcription in response to oestrogen. This analysis was taken one step further utilizing α-amanitin cell cycle synchronized MCF-7 cells (Métivier, R. *et al.* 2003). Three different transcription cycles were identified. During an initial transcriptionally unproductive and shorter cycle, the nucleosome is opened and histones H3 and H4 become modified to steady state levels. Only in the following two productive cycles where histone acetylation and dimethylation increases dramatically, are p68 RNA helicase, p160, HAT and a host of other cofactors recruited to phosphorylate RNA Pol II and initiate trancription. A different set of cofactors was shown to be involved a SWI/SNF complex to deacetylate histones and dissociate the ERα from the promoter to repress transcription.

The kind of cofactors and the sequence of events differs according to the promoter and cell type. However, general features are thought to apply to the principle of: transcriptional regulation and therefore other nuclear receptors (Glass,

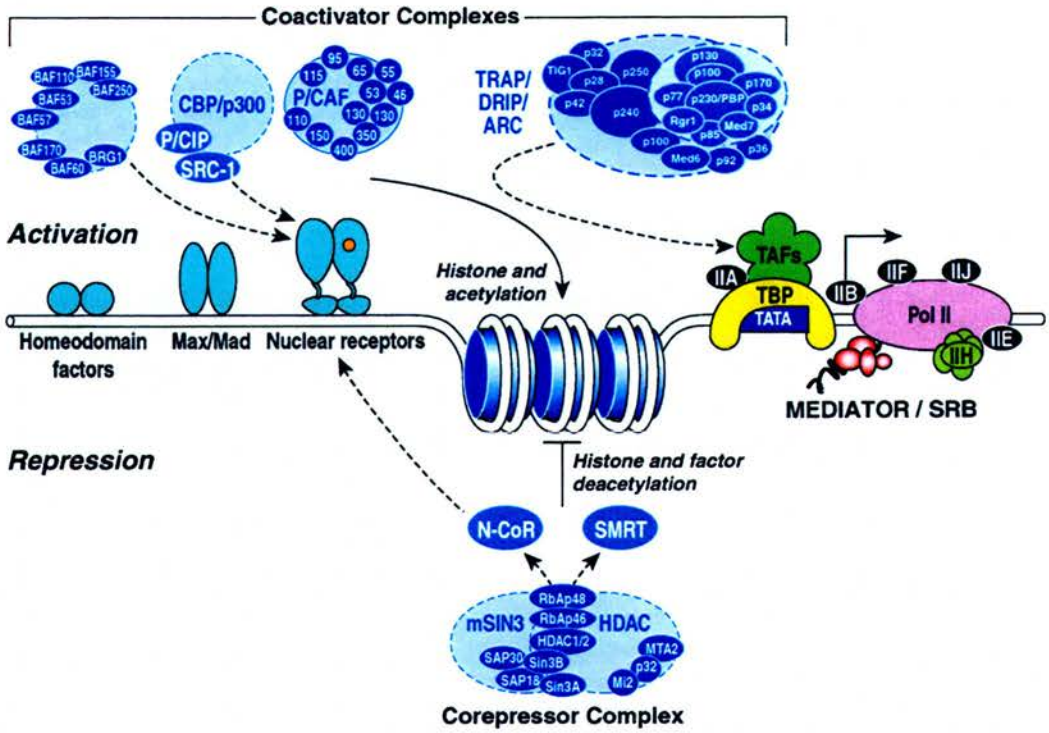


Figure 1.18: Schematic representation of coactivator and corepressor complexes in classical NR mediated transcription activation. ATP dependent coactivator complex BRG1; HAT activity possessing complexes CBP/p300, P/CAF and p160. TRAP/DRIP complex enhances chromatin independent transcription. Corepressors NCoR and SMRT attract a corepressor complex containing mSIN3 and HDACs (source: Glass, C.K. and Rosenfeld, M.G. 2000)

C.K. and Rosenfeld, M.G. 2000) (see figure 1.18). The ligand such as oestrogen enters the nucleus via passive diffusion or active mediated transport to bind to the receptor. The ligand unbound receptor resides in the nucleus as well as the cytosol most likely stabilized by either a protein complex containing heat shock protein 90 (hsp90), a chaperone known to be involved in folding and activating of NR, or by corepressors such as NCoR and SMRT. After hormone binding, geneactivation involves roughly two steps:

A. The liganded receptor undergoes a conformational change forming homo- or heterodimers and dissociates from its chaperones. Subsequent binding of ER to the ERE in the promoter attracts HAT containing coactivators complexes including p160, PBP/p300and pCAF components. HAT complexes modify local chromatin structure, acetylating lysine residues in histones H3 and H4 to relieve chromatin repression.

B. The DNA is now readily accessible for additional, basal transcription factors. A transcription preinitiation complex is assembled at the transcription start site, the TATA box of the promoter involving the recruitment of TATA box binding protein (TBP), TBP-associated factors (TAFs) and RNA II.

Alternative activations such as ERs interaction with other DNA bound transcription factors for example AP-1 or Sp1 may follow a similar model where ER-associated coactivator complexes use protein-protein interactions to set the stage for the transcription preinitiation complex.

1.4 Antioestrogen resistance

1.4.2 Antioestrogens: mechanism of action

Tamoxifen has been the most widely used antioestrogen for many years. About two-thirds of ER-positive metastatic breast tumours respond to this form of treatment (Dowsett, M. and Howell, A. 2002). Binding of tamoxifen results in altered receptor dimerization and prevents AF-2 mediated transactivation while AF-1 mediation remains functional (Dowsett, M. and Howell, A. 2002). In addition, tamoxifen induced receptor conformation results in changed coactivator and corepressor availability for transcription complex assembly influencing transcriptional outcome. The differential effect on the receptor activation domains gives rise to tamoxifen's partial agonism in addition to its antagonistic actions. Its two major metabolites *N*-desmethyltamoxifen and *trans* –4 hydroxytamoxifen have a similar affinity for the ER as 17-β oestradiol (Osborne, C. K. *et al.* 2000). Oestrogens are thought to agonize tumor growth mainly by stimulating progression from G_1 to S phase, increasing cell numbers in S and G_2/M stages, as well as activating non- cycling G_0 cells into the cell cycle (Parl, F.F. 2000(d)). On the other hand, tamoxifen, like many antioestrogens, is thought to be cytostatic and slow cell proliferation by

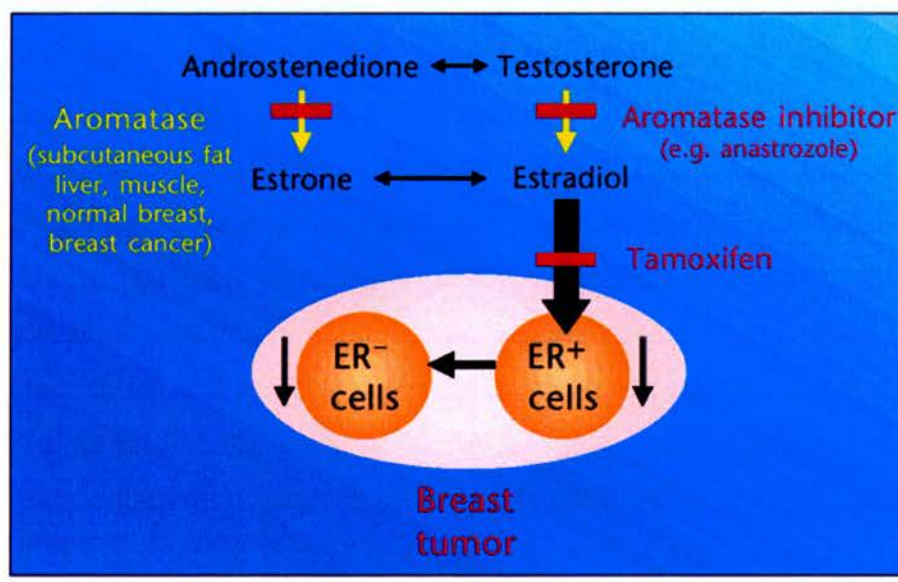


Figure 1.19: Mechanism of action of classical antieostrogens such as tamoxifen and of aromatase inhibitors such as anastrozole (source: Dowsett, M. and Howell, A. 2002).

blocking progression from the active G_0/G_1 cell cycle phase. There is also evidence that it might also have the ability to induce apoptosis and cause cell death. Antioestrogens could initially show cytostatic and then progress to cytotoxic effects (Clarke, R. *et al.* 2003). Oestradiol analogues ICI164,384 and ICI 182,780 (Fulvestrant/Faslodex) are steroidal ER inhibitors regarded as pure antagonists because of their lack of oestrogenic activity (Osborne, K. *et al.* 2000). ICI 182,780 is thought to inhibit ER-AF-1 and AF-2 mediated transactivation and stimulate ER degradation. The ER binding affinity of ICI 182,780 is similar to that of oestrogen but 100 fold stronger than tamoxifen.

A different class of antieoestrogens, steroidal and non-steroidal aromatase inhibitors such as Formestane or Letrozole, do not directly interfere with oestrogen binding to the receptor but prevent the synthesis of oestrogens by directly inhibiting the conversion of androgens into oestrogen, a step catalyzed by aromatase enzymes, to withdraw the hormone from the environment (Haynes, B.P. *et al.* 2003, Miller, W.R. 2004) (see figure 1.19). Steroidal and non-steroidal aromatase inhibitors both associate with the substrate binding site of the enzyme but inactivate by different mechanisms (Howell, A. *et al.* 2001). Steroidal compounds (type I) bind irreversibly to the active site of the aromatase enzyme, competing with the natural androgen to inactivate the enzyme. Nonsteroids (type II) interact with the active site in a reversible manner but also associate with the heme molecule prothetic group (cytochrome P_{450} part) of the enzyme. The reversible association requires the continued presence of the inhibitor to prevent aromatase function. Different mechanisms of action for aromatase inhibitors might be the basis for their differential potency and potential non-cross-resistance.

Strictly speaking, 'classical' antioestrogens like tamoxifen are not plain antioestrogens. More appropriately the term Selective Estrogen Receptor Modulators (SERMs) is now used for such agents, which directly bind the ER to interfere with gene transcription but may also have agonistic characteristics in the same or other tissues. Separate categories are assigned to pure antioestrogens, also called Selective Estrogen Receptor Downregulators (SERDs), and aromatase inhibitors (Johnston, S.R.D. 2004).

1.4.3 Mechanisms of antioestrogen resistance

Interestingly, tamoxifen treatment beyond five years has not proven to be beneficial. The drug shows an increase in oestrogenic side effects and loss of anti-tumour action (Lewis, J.S. *et al.* 2004). This is thought to be predominantly due to the development of antioestrogen resistance. Most tumours initially responsive to tamoxifen eventually become resistant to the drug. Resistant phenotypes are one of two types: intrinsic/de novo or acquired. The former is a pre-existing resistance in mostly ER -negative but also ER -positive tumours whereas the latter refers to ER – positive tumours developing drug resistance over time. Efforts have been made to understand the molecular mechanisms behind the evolution of antihormonal resistance. It has been shown that the loss of ER expression is uncommon and therefore not a prerequisite to develop ICI 182, 780 and tamoxifen resistance (Brünner, N *et al.* 1993 (a) and 1997). Most tumours remain ER+ and some responsive to pure antioestrogens indicating that the ER remains intact but loses its foremost position in E_2 dependent transcription. The cell is able to develop alternate pathways affecting apoptosis and proliferation functions. With the discovery of a second ER as well as several ER splice variants emerged a potential alternative route where a switch from ERα to ERβ mediated pathway might lead to resistance. Although both receptors show distinct functionalities, a change in receptor ratios reflecting phenotype progression has not clearly been demonstrated (Clarke, R. *et al.* 2003). Mounting evidence of altered coactivator and corepressor expression and activation of as well as involvement of other growth factor and cytokine pathways hint at a more complex shift in the signalling network of antioestrogen sensitivity and resistance.

A recent model for acquired resistance suggests a roughly three phase progression to antihormone resistance based largely on T47D and MCF-7 xenografts (Schafer, M.J. *et al.* 2003; Lewis, J.S. *et al.* 2004). During initial tamoxifen treatment, phase I sees oestrogen stimulation and anticipated SERM inhibition in breast tissues. Progression to phase II and III is the result of long-term treatment. In phase II any SERM as well as E_2 will maintain growth. After about 5 years, SERMs will continue to stimulate growth in Phase III but E_2 will become tumoricidal. The tumor is essentially able to grow independently. There is also evidence that E_2 not

only fails to stimulate growth after long-term antioestrogen treatment but also may induce apoptosis at physiological concentration. Estrogens are generally thought to agonize tumor growth by increasing cell numbers in S and G_2/M stages as well as reducing cells in G_0/G_1 stages to stimulate. As mentioned earlier, antioestrogens are thought to stimulate G_0/G_1 cell cycle arrest. This observation has been made in breast cancer cells long-term (1year) -deprived of oestrogen and designed to mimic prolonged antioestrogen treatment and hence, E_2 withdrawal.

Increasing attention has been drawn to potential cross-talk of the ER with cell surface signalling pathways in the development of antioestrogen resistance. Overexpression of the tyrosine kinase gene HER2/neu (erbB-2) has been correlated with a shorter overall and disease-free survival and associated with a reduced ER expression. Interestingly, experiments have also shown that overexpression of HER2/neu in breast cancer cells in vitro results in tamoxifen resistance. This can be reversed by blocking the HER2/neu pathway (Kurokawa, H. *et al.* 2000). A recent study confirms that E_2 stimulated tumors become tamoxifen and E_2 stimulated after longterm tamoxifen treatment (Schafer, M.J. *et al.* 2003). The HER2/neu protein and

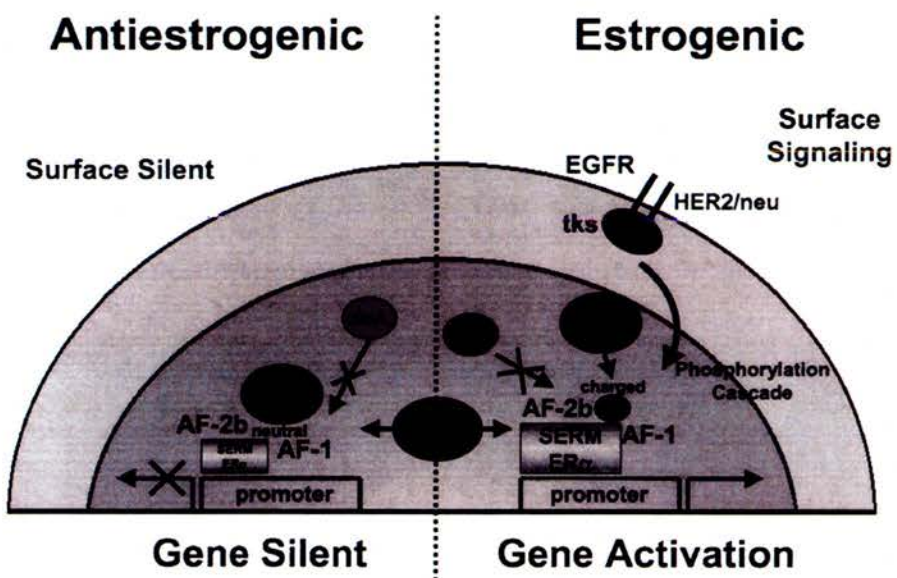


Figure 1.20: Proposed model of the integrated mechanism for the target site-specific action of SERMs. SERMs act as antioestrogens in surface silent cells (i.e. cells that express low levels of growth factor receptors) resulting in dominant co-repressor activity that retains the antioestrogenic activity of SERMs. However, when growth factor receptors are expressed at the cell surface resulting in dominant co-activator activity, the SERM:oestrogen receptor complex becomes transcriptionally active (source: Schafer, M.J. *et al.* 2003).

mRNA as well as the ER and PR protein expression increases. A model for the integrated mechanism of acquired antioestrogen resistance has been proposed (see Fig 1.20). Antioestrogenic action might be linked to breast cancer cells with absent HER2/neu cell surface signalling where corepressors are recruited by the tamoxifen bound receptor. In the resistant and tamoxifen stimulated phase, cells might have been selected for their increased cell surface signalling. Alternative pathways phosphorylate the ER-tamoxifen complex and coactivators take precedence to stimulate ER mediated transcription. In this model, both, alternative pathways and the ER are required for the development of antioestrogen resistance. The ER, therefore remains to play a pivotal role during acquired antihormone resistance. However, the molecular mechanism of ER signalling in response to endocrine agents appears to be a highly adaptable multifactorial network of several pathways.

1.4.3 Model systems

(i) Breast cancer cells

A number of breast cancer cell line and xenograft models have been developed in recent years attempting to mimic the oestrogen independent phenotype and the clinical setting of antioestrogen resistance acquisition. Despite the intense interest in suitable laboratory models, there is only a small core of about 100 breast cancer cell lines that have been consistently used for research (comprehensive review by Lacroix, M. and Leclercq, G. 2004). Most common is the use of ER and PR positive MCF-7 and T-47D. A large panel of breast carcinoma cells designated MDA-MB was established by Cailleau, R. and collegues (1987). This includes MDA-MB-231 cells, an epithelial adenocarcinoma cell line isolated from a pleural effusion. MDA-MB-231 cells are ER and PR negative, show invasive

properties *in vitro* and are able to form tumours *in vivo* (Garcia, M. *et al.* 1992; Thompson, E.W. *et al.* 1992). Other individual cell lines have been selected for specific characteristics such as NR status or HER2/neu overexpression as well as their tumour type to represent a particular clinical phenotype. One example is the MDA-MB-330 line, which has been isolated from a relatively rare lobular carcinoma (Cailleau, R. *et al.* 1978). The differentiated extraction of cells from surrounding

stroma and their viability long term have made the establishment of cell lines difficult.

The HCC (Hamon Cancer Centre) series is an example of a series of related breast cancer cell lines. Related cell lines have either been obtained from the same patient or in the HCC series case, been isolated in the same laboratory (Gazdar, A.F. *et al.* 1998). This series was an attempt to isolate multiple cell lines from tumours with distinct geno- and phenotypes for comparison between the *in vitro* and *in vivo* material. Breast cancer variant lines are selected from their wild type predecessors for growth in media containing specific growth factors or antioestrogens. The well-studied HMT-3522 line was originally established from a fibrocystic lesion as a non-malignant ER- epithelial line dependent on the presence of growth factor EGF (Briand, P. and Lykkesfeldt, A.E. 2001). Multiple sublines have been developed from the wild type cells to establish a model to study breast carcinogenesis. Malignant HMT-3522/S-2 and HMT-3522/T4-2 cells are EGF inhibited and EGF unresponsive, respectively. A separate subline, HMT-3522F9, is comprised of EGF dependent but ER positive cells. Of particular interest are also cell lines containing particular germline mutations. The ER positive and PR negative HCC1937 cell line has shown to carry a BRCA1 mutation (Tomlinson, G.E. *et al.* 1998).

There has always been debate at to how well cell models represent the tumours from which they have been derived. Breast cancer tumours are known to be particularly heterogeneous and progress pathologically and clinically towards a metastatic phenotype. Although breast cancer cell lines have also shown considerable heterogeneity, it has been questioned whether pure and clonal populations of breast cancer cells *in vitro* characterize changes in the clinical material. Complex DNA copy number changes have been demonstrated in multiple breast cancer cell lines and the corresponding primary tumours with genomic hybridisation experiments (Larramendy, M.L. *et al.* 2000). Results also reveal that almost all aberration found in tumours are present in the cell lines although cell lines do show additional changes. Long term culturing of breast cancer cells may lead to a deceptive selection of cells based on culturing conditions that do not mirror *in vivo* settings. Again, phenotypic or genotypic changes might not develop in cell lines parallel to tumour material as a result. It has been known that the same cell lines grown in various

laboratories under differing conditions can give raise to multiple subpopulations. Variations for separate MCF-7 cell stocks include proliferative response to 17β-oestradiol and DNA copy number changes (Jones, C. *et al.* 2000).

(ii) The MCF-7 and MCF-7 resistant cell lines

The model system used in this study originates from MCF-7 cells isolated from a pleural effusion in a postmenopausal patient with metastatic breast cancer in 1973 (Soule, H.D. *et al.* 1973). This cell line has since been well established for use in breast cancer research (reviewed in Levenson, A.S. and Jordan, V.C. 1997). MCF-7 cells have been shown to be multiple receptor positive including glucocorticoid, progesterone, androgen and oestrogen receptors. Functional ERs have been identified in the nucleus as well as the cytosol where the majority of ERs reside. MCF-7 cells have been used to identify potential prognostic markers such as cathepsin D or pS2 (Augerau, P. *et al.* 1988; Masiakowski, P. *et al.* 1982). The cells have been of value to study the mechanisms of crosstalk between E_2 stimulated growth and TGFα/β or IGFI/II growth factor pathways. MCF-7 cell proliferation is induced by oestrogen and TGFα although stimulation is weaker and TGFα alone does not result in E_2 independent cell growth (Clarke, R. *et al.* 1989). Despite their role in MCF-7 cell growth, growth factors like TGFα and their receptors themselves appear to be expressed in low levels (Levenson, A.S. and Jordan, V.C. 1997 and references within). Several antioestrogens inhibit cell growth. This includes tamoxifen which blocks the G_1 phase of the cell cycle (Osborne, C.K. *et al.* 1983 and others).

A variety of sublines have been developed including the antioestrogen variant lines MCF-7/MIII, MCF-7/LCC-1, MCF-7/LCC-2 and MCF-7/LCC-9 as illustrated in figure 1.21. To generate hormone independent but antioestrogen phenotypes assumed to be present in may postmenopausal women, MIII cells were directly selected following inoculation of MCF-7 cells into overiectomized NCr *nu/nu* athymic nude mice. Generally these cells require the addition of exogenous oestrogen but a subpopulation of cells grew giving rise to the MIII cell line. This type of selection assures cell proliferation in an endocrine environment of low oestrogen serum levels comparable to postmenopausal women (Clarke, R and Brünner, N. 1995). It gave rise to an oestrogen independent cell line still responsive to all major classes of antioestrogens *in vitro*. Further selection by *in vivo* passaging

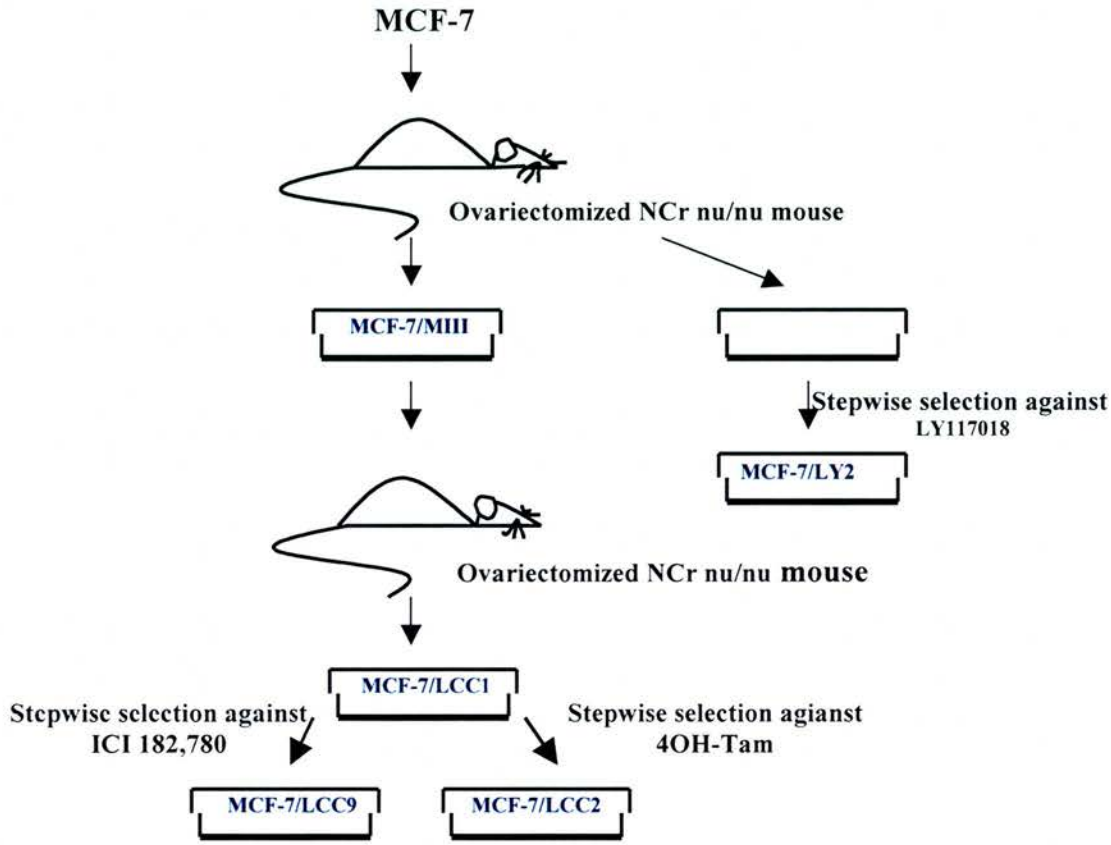


Figure 1.21: Derivation of the MCF-7 variant cell lines MIII, LCC-1, LCC-2 and LCC-9 (source: Brünner, N. *et al.* 1997).

of MIII cells resulted in LCC-1 cells, which retain hormone and antihormone characteristics of its predecessor (Brünner, N. *et al.* 1993 (a)). LCC-1 cells exhibit a reduced time to tumour formation *in vivo*. A stepwise *in vitro* selection against 4-OH-tamoxifen and ICI 182,780 from LCC-1 cells established LCC2 and LCC9 cells, respectively (Brünner, N. *et al.* 1993 (b) and 1997). Both cell lines show relative oestrogen resistance. Interestingly, LCC-2 cells are *in* vitro and *in* vivo tamoxifen resistant but remain responsive to steroidal antioestrogens such as ICI 182,780. On the other hand, LCC-9 cells acquire an *in vitro* and *in vivo* resistance to triphenylethylenes alongside with their resistance to ICI 182,780. Brünner and collegues report that the three resistant lines express the oestrogen receptor protein levels similar to the wild type MCF-7 cells. MCF-7, LCC-1 and LCC-2 cells exhibit E_2 inducible PR protein expression. Between the four related cell lines, LCC-9 cells show the highest level of PR protein but expression is unaffected by oestrogen. This

sequential cell selection was based on the concept that morphological changes would mirror a malignant progression in breast cancer. It was hypothesised that this model could be used to study the clinical progression of hormone independence and antioestrogen resistance.

LY2 cell were established in a separate branch from MCF-7 cells. As the name suggests, they were selected by passaging against increasing concentrations of the antioestrogen LY 117,018 (Bronzert, D.A. *et al.* 1985)(figure 1.18). LY2 cells are reported to proliferate rapidly without the presence of E_2 but retain a relative E_2 responsiveness. In addition to LY 117,018, a benzothiophene, this cell line is also resistant to triphenylethylenes tamoxifen and 4-OH tamoxifen as well as ICI 164,384 (Bronzert, D.A. *et al.* 1985; Clarke, R. *et al.* 1989). LY2 cells were found to not express PR protein either with or without E_2 (Bronzert, D.A. *et al.* 1985). Levels of ER protein expression are similar in MCF-7 and LY2 cells although a distinction was made between cytosolic and nuclear ER expression. Similar to MCF-7 cells, transcription of E_2 target genes CTSD and pS2 was stimulated by the hormone suggesting ER regulation (Mullick, A. and Chambon, P. 1990). The levels of ER protein extracted from the nucleus was reported to be much lower. The LY2 cell line was therefore designed as a model for ER positive breast cancer cross –resistant to different groups of antieostrogens. Similar to the LCC line, it was hoped this model could help reveal the molecular mechanism of the development of antieostrogen resistance. In contrast to MCF-7 cells, LY2 cells are nontumorigenic and consequently can only be used in *in vitro* experiments (Clarke, R. *et al.* 2001).

1.5 Aims and Objectives

This study aimed to investigate changes in the activation process in MCF-7 variant cell lines with a view to identifying potential mechanisms that may help explain acquired endocrine resistance by

(i) Confirming the growth phenotypes of the breast cancer cell line MCF-7 and MCF-7 variants for their use as model systems in this study. In particular, the growth response to oestrogen and the antioestrogen tamoxifen has been determined.

(ii) Analyse transcription and translation of the ER and PR as well as coactivators and corepressors in response to endocrine treatment using quantitative reverse transcription–polymerase chain reaction and western blot analysis.

(iii) Investigating a series of oestrogen-regulated genes pS2, MYC and cathepsin D to ascertain which expression pattern changes best reflected oestrogen-regulated growth.

(iv) Exploring the activation process at the pS2 promoter using ChIP technology to identify ERα binding, p160 family protein recruitment and gene activation as indicated by H4 acetylation.

Hypothesis

Hormone resistant human breast cancer cell lines show differential E_2 target gene expression and ER mediated gene transcription complex assembly. This thesis set out to explore these possibilities. Changes of this kind may contribute to the development of endocrine resistance.

Chapter 2: Results

2.1 Characterization of cell lines as models for endocrine resistance

2.1.1 Introduction

To explore mechanisms of oestrogen and antioestrogen resistance, model systems were employed. Two groups of cell lines derived from the ER-positive MCF-7 cell line were selected –the LCC-1/LCC-2/LCC-9 series developed by Clarke, R. and collaborators (Brünner, N. *et al.* 1993 and 1997) and the LY2 cell line developed by Bronzert, D.A. (1985). The MDA-MB-231 cell line was selected as an example of an ERα -negative model. This part of the study was designed to validate characteristic growth patterns exhibited by this panel of breast cancer cells. MCF-7 cells were plated in phenol red containing media (complete medium) for 24h allowing cells to attach to the plastic. To remove any oestrogenic stimulation, cells were washed in PBS and left in phenol red free medium (reduced medium) for 48h. LCC-1, LCC-2, LCC-9, LY2 and MDA-MB-231 cells were plated immediately in phenol red free medium and left for 24h. Cells were then maintained in reduced medium and supplemented with 10^{-9}M E_2, 10^{-6}M tamoxifen or 10^{-9}M E_2 and 10^{-6}M tamoxifen. The medium was replaced every two days. Duplicate cell counts were measured using a Coulter counter on days 0, 2, 4 and 6 where day 0 represents counts on the day endocrine agents are added to the media (before supplementation). Cells not treated were used as controls to monitor cellular growth.

Cells were photographed on day 2 to illustrate phenotypic changes supporting the observed differences between cell lines and different treatement groups. This work was done as an accompaniment to the detailed characterization experiment using cell counts.

2.1.2 Parental MCF-7 cell line

MCF-7 cells demonstrate a distinct morphology of polygonal epithelial cancer cells (figure 2.1). Cells are spread-out, strongly attached to the plastic and readily form cell-cell contact. Once well defined colonies are formed, the proliferation rate increases dramatically. Upon oestrogen addition, the appearance of the cells changes markedly. Cells become spherical and less adherent. Cells remain in tight colonies and proliferate at a much higher rate. The morphological effect can be observed to a lesser extent in cell cultures exposed to E_2 and tamoxifen.

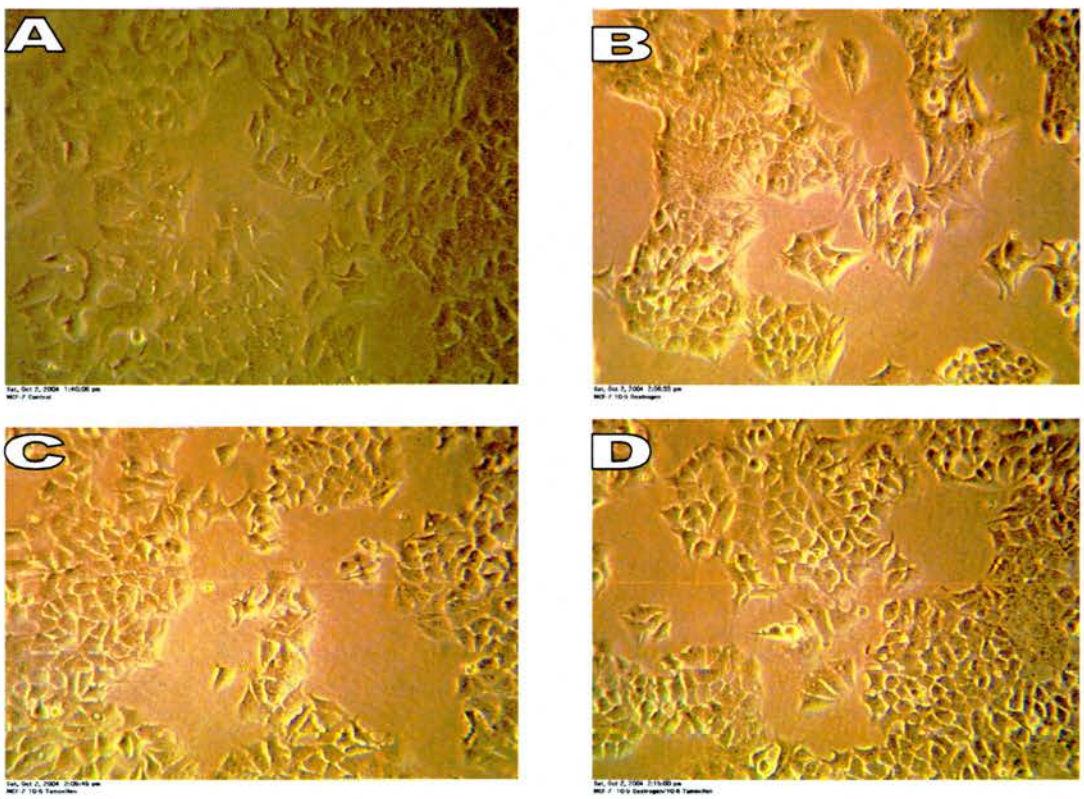
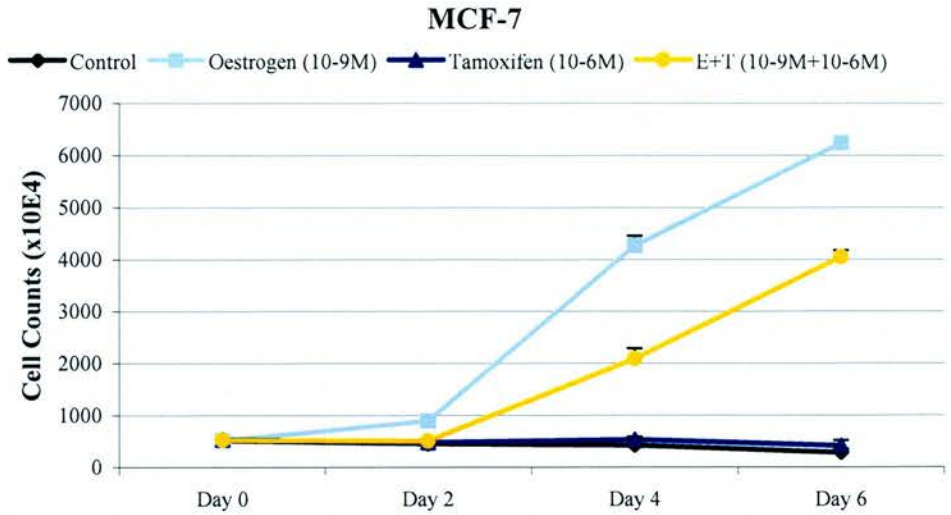


Figure 2.1: Morphological comparison of MCF-7 cells in living monolayers. Cells have been plated in phenol red containing DMEM supplemented with 5%stripped FCS, 1%Pen/Strep and 2mM glutamine for 24h followed by phenol free DMEM for 48h (A) Media was supplemented with 10^{-9}M E_2 (B); 10^{-6}M tam (C) or 10^{-9}M E_2 and 10^{-6}M tam (D) for 48h. Cells were photographed using an inverted Zeiss microscope at 200x.



	C/E_2	C/Tam	C/E_2+Tam	E_2/ E_2+Tam
Day 0	n.s.	n.s.	n.s.	n.s
Day 2	**	n.s.	n.s.	**
Day 4	**	n.s.	**	**
Day 6	**	n.s.	**	**

Figure 2.2: Cell line characterization of MCF-7 cells. Cells were plated in complete media for 24h and maintained in reduced media for 48h before treatment. Cells were then left untreated (control group), treated with 10^{-9}M E_2; 10^{-6}M tam; or 10^{-9}M E_2 and 10^{-6}M tam. Cells were counted on day 0 (72h after plating/ day of treatment start) and day 2/4/6 using a coulter counter. Cell counts of triplicate samples and duplicate counts for each time point in each treatment group are expressed. Error bars=STD. Representitive experiment is shown of at least two experiments carried out. Table shows significant variance between treatment groups and control group as well as E_2 and E_2+Tam determined by one-way ANOVA and Tukey-Kramer multiple comparison test: n.s. = not significant, *=$p<0.05$, **=$p<0.01$.

Changes in growth pattern in response to endocrine agents observed under the microscope were also established with cell counts (Figure 2.2). When MCF-7 cells are maintained in phenol red free media, proliferation ceases after 24h although most existing cells remain alive (1.8 fold decrease). Oestrogen markedly stimulated cell growth with a most dramatic increase in cell number by day 6 (12.1 fold). Counts of the cell group treated with tamoxifen are very similar to counts of the control group. The cell number remains largely unchanged throughout day 2 and day 4 and decreases marginally by day 6 (1.3 fold). In combination, tamoxifen is able to partially represses the oestrogen stimulated proliferation. When day 6 is reached, the cell number has increased only 7.7 fold, about half the increase observed in the oestrogen treated group. These data present MCF-7 cells as a classical oestrogen and tamoxifen sensitive cell line where oestrogen stimulates and tamoxifen antagonizes oestrogen-stimulated cell growth.

2.1.3 MCF-7 variant cell lines

(i) MCF-7/LCC-1 cells

LCC-1 cells are clearly distinguishable from the parental MCF-7 cell line (Figure 2.3). Cells appear in a more circular shape. Adherence is not as strong although colony formation is apparent. Proliferation rate appears more rapid than MCF-7 cells. This was again confirmed using coulter counter analysis (Fig 2.4). In stark contrast to the parental line, cell numbers in the control group have quadrupled by 48h. Cells remain in active proliferation throughout the course of the experiment (17.4 fold by day 6). The growth pattern of cells treated with tamoxifen is comparable to the control group. Oestrogen steadily increases cell growth but to a lesser extent than the effect seen in MCF-7 cells. The cell number has increased 22.6 fold by day 6; that is 1.4 fold further than the control group has reached at that point in the experiment. The addition of tamoxifen did not alter the stimulation of

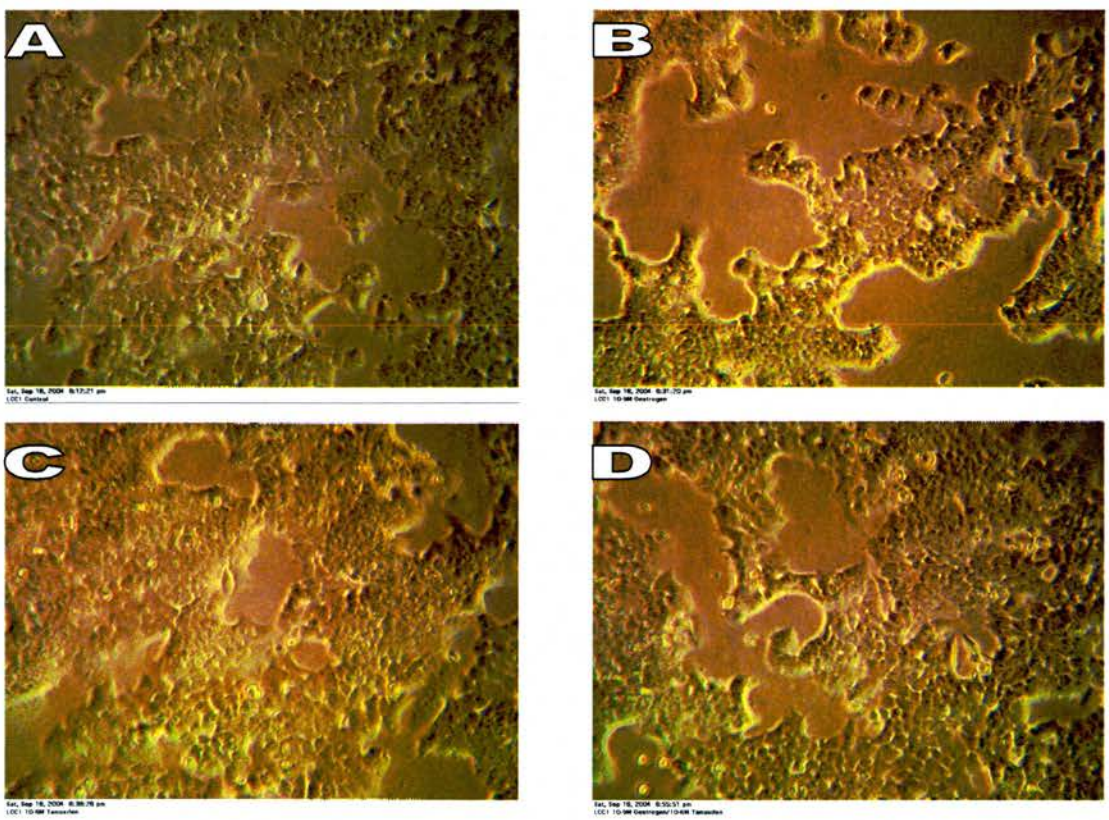
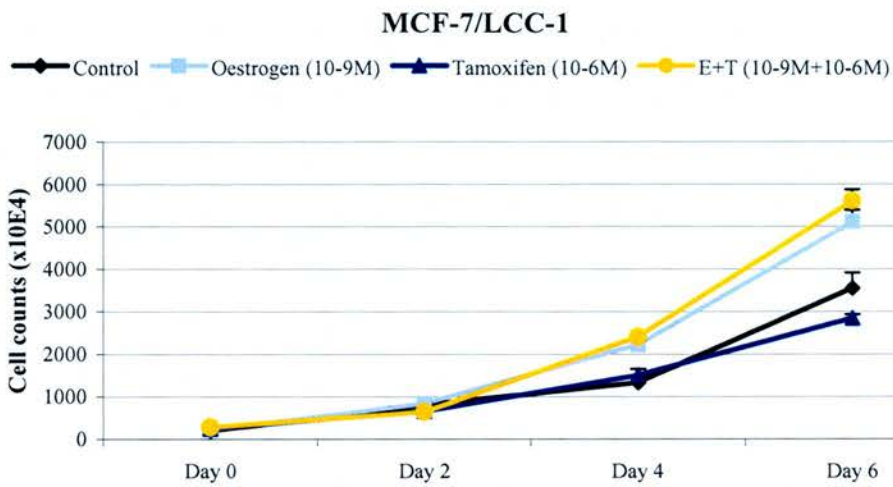


Figure 2.3: Morphological comparison of LCC-1 cells in living monolayers. Cells have been plated in phenol red free DMEM supplemented with 5%stripped FCS, 1%Pen/Strep and 2mM glutamine for 24h(A). Media was supplemented with 10^{-9}M E_2 (B); 10^{-6}M tam (C) or 10^{-9}M E_2 and 10^{-6}M tam (D) for 48h. Cells were photographed using an inverted Zeiss microscope at 200x.

oestrogen in this cell line. The two treatment groups exhibit almost identical growth phenotypes. These results demonstrate that LCC-1 cells grow rapidly when maintained in reduced media conditions. However, cells show a markedly reduced oestrogen and tamoxifen sensitivity as compared to MCF-7 cells.



	C/E_2	C/Tam	C/E_2+Tam	E_2/ E_2+Tam
Day 0	n.s.	n.s.	**	n.s.
Day 2	n.s.	**	**	**
Day 4	**	n.s.	**	n.s.
Day 6	**	*	**	n.s.

Figure 2.4: Cell line characterization of MCF-7/LCC-1 cells. Cells were plated in reduced media for 24h before treatment. Cells were then left untreated (control group), treated with 10^{-9}M E_2; 10^{-6}M tam; or 10^{-9}M E_2 and 10^{-6}M tam. Cells were counted on day 0 (24h after plating/ day of treatment start) and day 2/4/6 using a coulter counter. Cell counts of triplicate samples and duplicate counts for each time point in each treatment group are expressed. Error bars=STD. Representitive experiment is shown of at least two experiments carried out. Table shows significant variance between treatment groups and control group as well as E_2 and E_2+Tam determined by one-way ANOVA and Tukey-Kramer multiple comparison test: n.s. = not significant, *=$p<0.05$, **=$p<0.01$.

(ii) MCF-7/LCC-2 cells

In a manner similar to LCC-1 cells, the LCC-2 cell line grows as rounded cells in colonies less tightly fixed to the surface of culture dish compared to the MCF-7 cells (figure 2.5). Colonies appear to lift off the plastic forming floating clusters rather than a single layer spreading across the plastic. The number of individual cells floating in culture media becomes considerable. This is even more evident when cells are exposed to E_2 alone or in combination with tamoxifen. Very little difference in appearance is observed when cells are treated with tamoxifen alone. Unlike MCF-7 and LCC-1 cells, all treatment groups of LCC-2 cells show

very similar growth pattern (Figure 2.6). LCC-2 cell proliferate rapidly in reduced media with the cell number doubled in less than 48h as seen with LCC-1 cells. Relative to MCF-7 cells oestrogen and tamoxifen have very small effects on cell proliferation. At day 6 all groups show a similar increase in cell number (control 14.4 fold, E_2 11.6 fold, tam 13.6 fold, E_2 and tam 13.6 fold). These results indicate that LCC-2 cells are oestrogen and tamoxifen resistant.

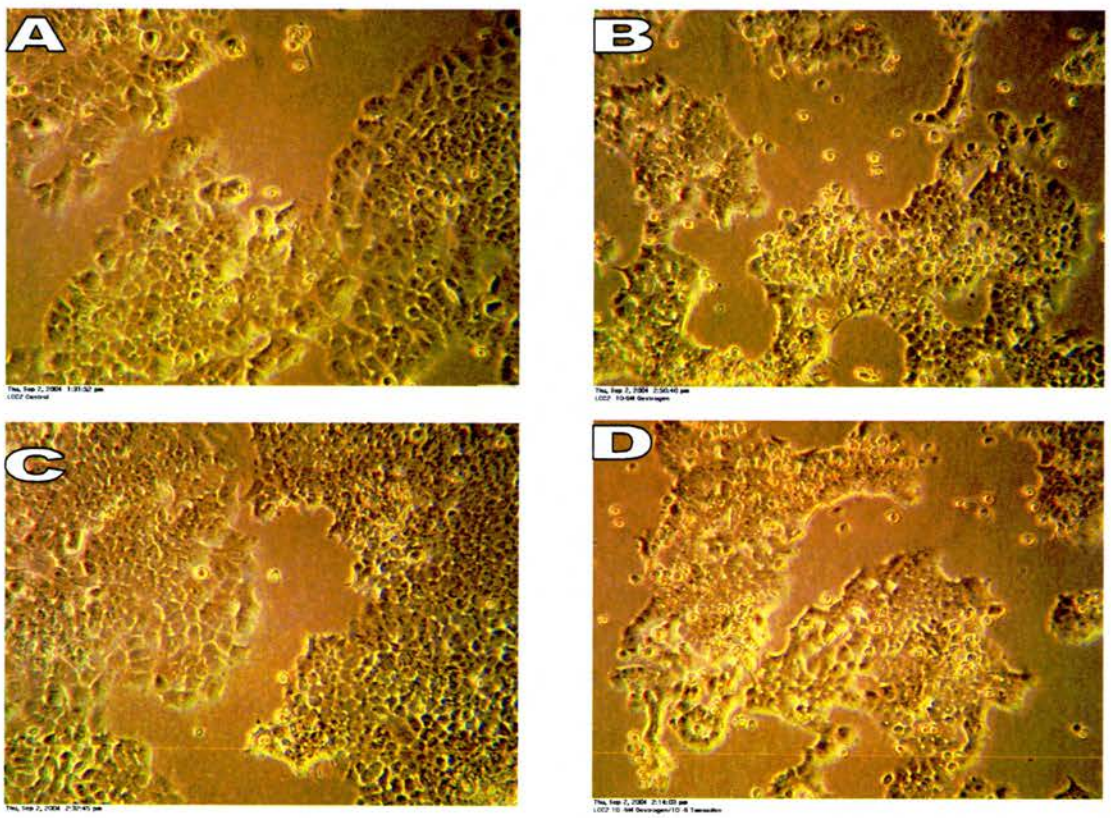
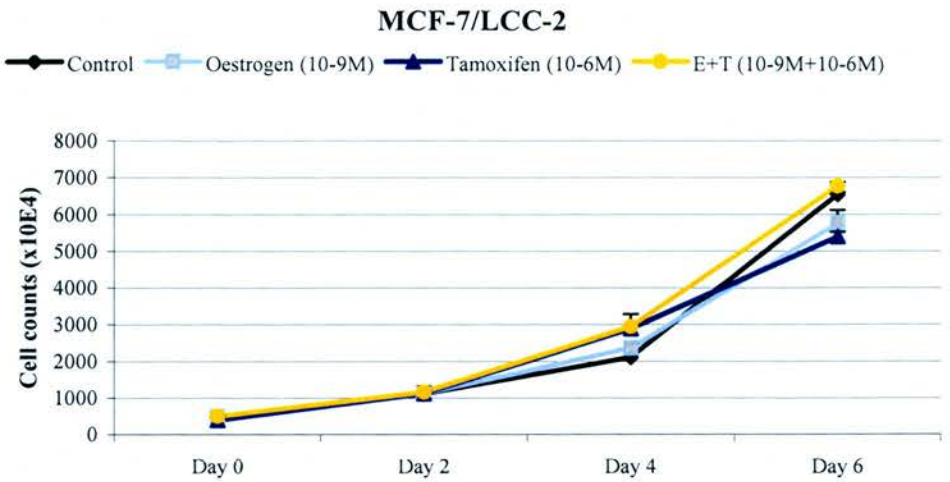


Figure 2.5: Morphological comparison of LCC-2 cells in living monolayers. Cells have been plated as LCC-1 line (see Figure 2.1.2). Media was supplemented with 10^{-9}M E_2 (B); 10^{-6}M tam (C) or 10^{-9}M E_2 and 10^{-6}M tam (D) for 48h. Cells were photographed using an inverted Zeiss microscope at 200x.



	C/E_2	C/Tam	C/E_2+Tam	E_2/ E_2+Tam
Day 0	n.s.	*	n.s.	n.s.
Day 2	n.s.	n.s.	n.s.	n.s.
Day 4	n.s.	**	**	**
Day 6	**	**	n.s.	**

Figure 2.6: Cell line characterization of MCF-7/LCC-2 cells. Cells were plated in reduced media for 24h before treatment. Cells were then left untreated (control group), treated with 10^{-9}M E_2; 10^{-6}M tam; or 10^{-9}M E_2 and 10^{-6}M tam. Cells were counted on day 0 (24h after plating/ day of treatment start) and day 2/4/6 using a coulter counter. Cell counts of triplicate samples and duplicate counts for each time point in each treatment group are expressed. Error bars=STD. Representitive experiment is shown of at least two experiments carried out. Table shows significant variance between treatment groups and control group as well as E_2 and E_2+Tam determined by one-way ANOVA and Tukey-Kramer multiple comparison test: n.s. = not significant, *=$p<0.05$, **=$p<0.01$.

(iii) MCF-7/LCC-9 cells

The change in appearance in this series of cell lines is most evident under the microscope when comparing MCF-7 cells with LCC-9 cells (Figure 2.7). This cell line gives an appearance of round scattered cells growing in poorly defined colonies. Cell-cell contact appears less coherent. Upon plating, colony formation does not appear to be necessary for a rapid proliferation rate as seen in the parental line. Freely floating cells are present in all groups. Oestrogen treatment seems to promote detachment of the cells from the culture dish even further.

Growth characteristics for LCC-9 cells are very similar to LCC-2 cells. LCC-9 cells grow rapidly with a doubling time less than 48h. Proliferation in all groups is observed within the first 48h of the experiment (control 2.1 fold, E_2 2.5 fold, tam 2.5 fold, E_2 and tam 2.7 fold). Cell growth remains largely unaffected by treatment with oestrogen as well as tamoxifen indicating a resistance to both agents.

At day 6, cell numbers in all groups have increased by on average 12.3 fold, an increase that compares to E_2 treated MCF-7 cells (12.1 fold) or to the average of all groups of LCC-2 cells at this time point (13.3 fold).

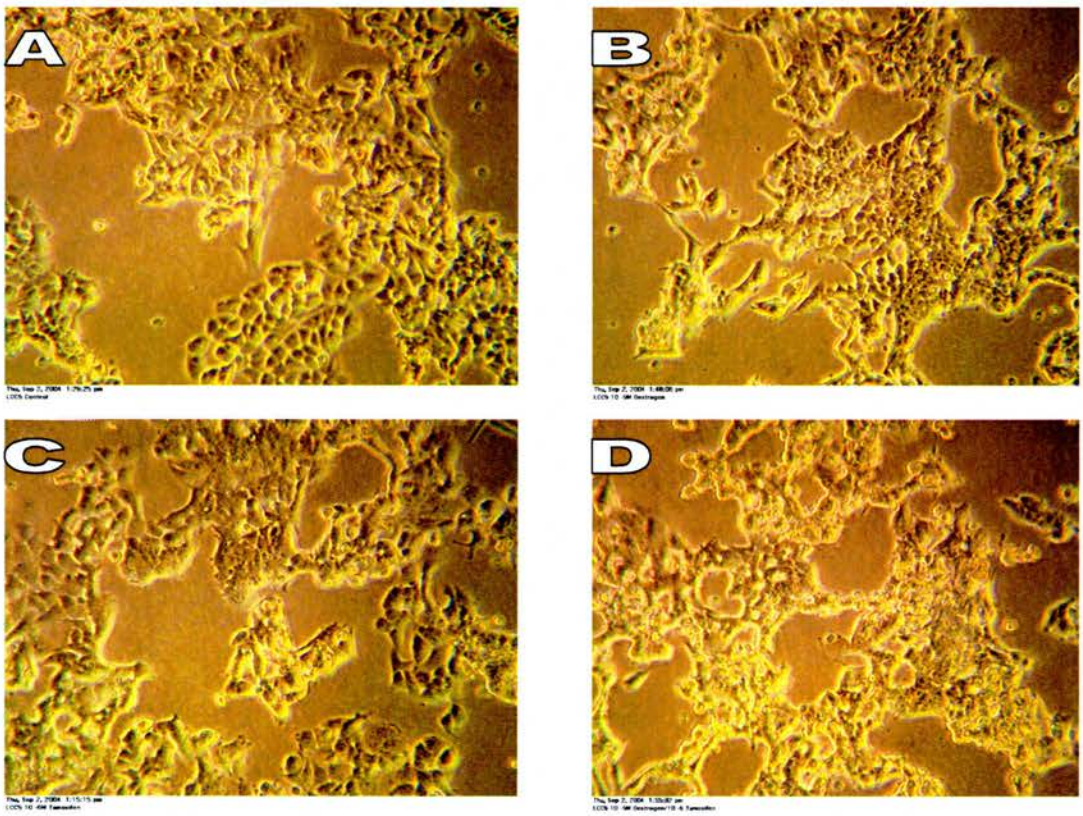
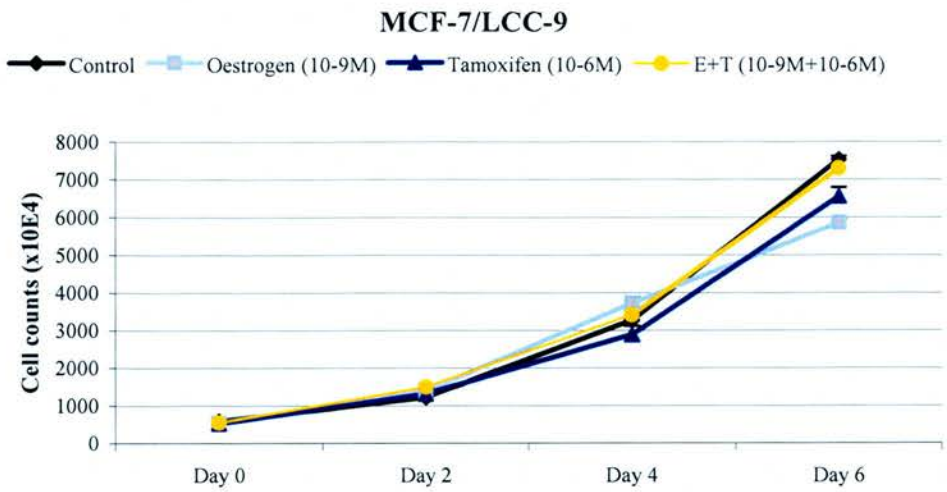


Figure 2.7: Morphological comparison of LCC-9 cells in living monolayers. Cells have been plated as LCC-1 line (see Figure 2.1.2). Media was supplemented with 10^{-9}M E_2 (B); 10^{-6}M tam (C) or 10^{-9}M E_2 and 10^{-6}M tam (D) for 48h. Cells were photographed using an inverted Zeiss microscope at 200x.



	C/E_2	C/Tam	C/E_2+Tam	E_2/ E_2+Tam
Day 0	n.s.	n.s.	n.s.	n.s.
Day 2	*	n.s.	**	n.s.
Day 4	n.s.	n.s.	n.s.	n.s.
Day 6	**	**	n.s.	**

Figure 2.8: Cell line characterization of MCF-7/LCC-9 cells. Cells were plated in reduced media for 24h before treatment. Cells were then left untreated (control group), treated with 10^{-9}M E_2; 10^{-6}M tam; or 10^{-9}M E_2 and 10^{-6}M tam. Cells were counted on day 0 (24h after plating/ day of treatment start) and day 2/4/6 using a coulter counter. Cell counts of triplicate samples and duplicate counts for each time point in each treatment group are expressed. Error bars=STD. Representitive experiment is shown of at least two experiments carried out. Table shows significant variance between treatment groups and control group as well as E_2 and E_2+Tam determined by one-way ANOVA and Tukey-Kramer multiple comparison test: n.s. = not significant, *=$p<0.05$, **=$p<0.01$.

Table 2.1 summarizes base line growth rates in reduced media comparing parental MCF-7 cells with the three variant lines. Numbers illustrate clearly that MCF-7 cells are unique in that they are oestrogen dependent and therefore unable to grow in E_2 deprived conditions (5% DCC in phenol red free DMEM). In contrast, LCC-1 cells grow consistently under these conditions with a doubling time of about 36h over the course of the experiment indicating a reduced need for the hormone. The third generation of cells derived from MCF-7 cells, LCC-2 and LCC-9 cells, demonstrate a relative oestrogen independence with similar doubling times of about 36h. MCF-7 cells are also explicitly different under the microscope when compared to the variant lines (Figure 2.9).

	MCF-7	LCC-1	LCC-2	LCC-9
Day 0	519	204	456	587
Day 2	457	809	1108	1221
Day 4	428	1335	2107	3265
Day 6	282	3548	6537	7530

Table 2.1: Comparison of average basal growth for MCF-7 and LCC-1/2/9 control groups as obtained in characterization experiments as explained above. Mean numbers from a typical experiment are shown.

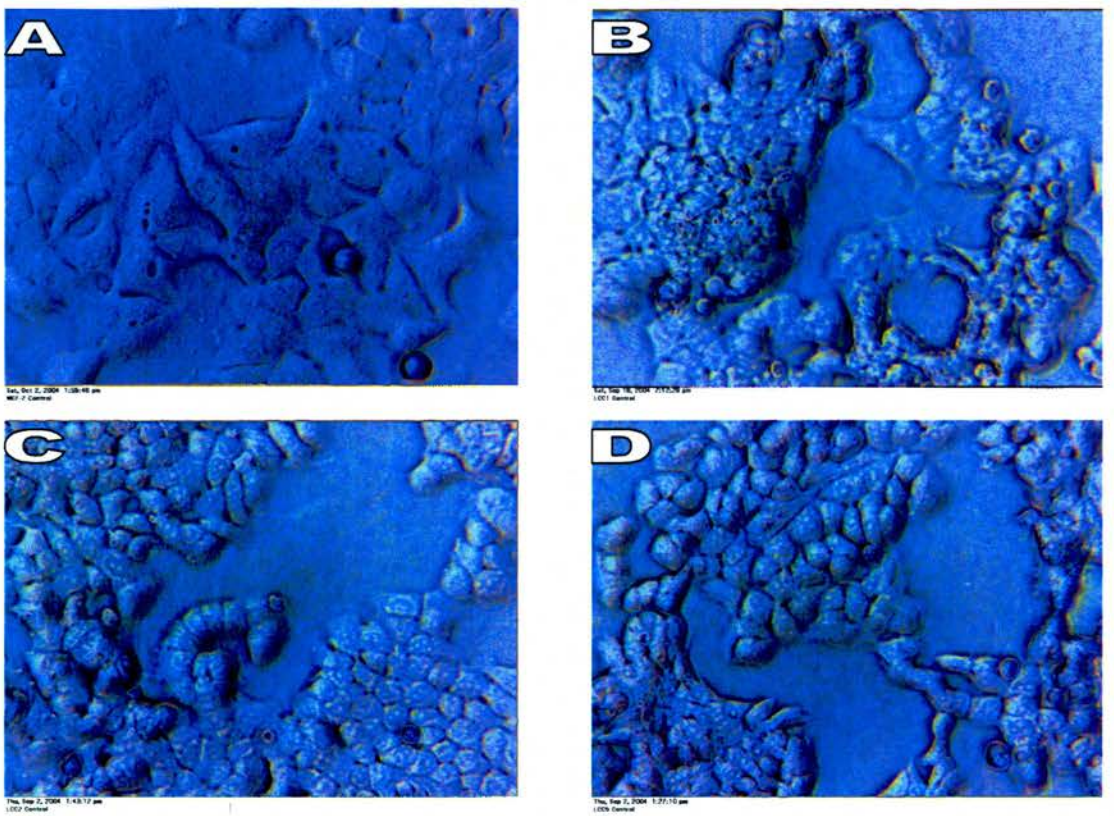
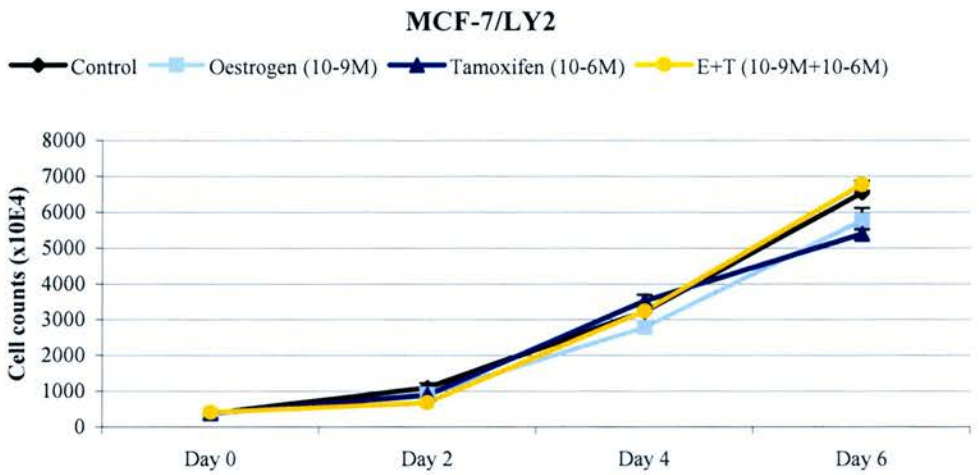


Figure 2.9: Morphological comparison of MCF-7 (A) with LCC-1 (B); LCC-2 (C) LCC-9 (D) Cells have been kept in reduced medium for 48h and photographed using an inverted Zeiss microscope at 200x.

(iv) MCF-7/ LY2 cells

This cell line was originally selected for its resistance to the antioestrogen LY 117018 (Bronzert, D.A. *et al.* 1985) but derived separately from the LCC variant lines. The growth experiment shows that LY2 cells are profoundly growth resistant to both tamoxifen and oestrogen (figure 2.10). The hormone fails to induce cell proliferation. Between day 0 and day 6 all treatment groups show a rapid cell doubling (control 17.5 fold, E_2 16.6 fold, tam 13.7, E_2 plus tam 16.6 fold). The doubling time is very similar to that of LCC-1, LCC-2 and LCC-9 cells (about 36h).



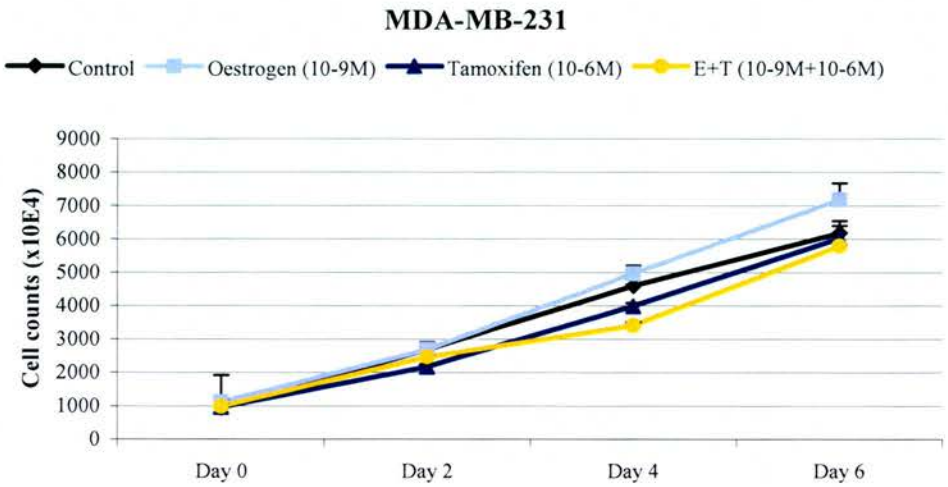
	C/E_2	C/Tam	C/E_2+Tam	E_2/ E_2+Tam
Day 0	n.s.	n.s.	n.s.	n.s.
Day 2	n.s.	n.s.	**	n.s.
Day 4	**	n.s.	n.s.	**
Day 6	**	**	n.s.	**

Figure 2.10: Cell line characterization of MCF-7/LY2 cells. Cells were plated in reduced media for 24h before treatment. Cells were then left untreated (control group), treated with 10^{-9}M E_2; 10^{-6}M tam; or 10^{-9}M E_2 and 10^{-6}M tam. Cells were counted on day 0 (24h after plating/ day of treatment start) and day 2/4/6 using a coulter counter. Cell counts of triplicate samples and duplicate counts for each time point in each treatment group are expressed. Error bars=STD. Representitive experiment is shown of at least two experiments carried out. Table shows significant variance between treatment groups and control group as well as E_2 and E_2+Tam determined by one-way ANOVA and Tukey-Kramer multiple comparison test: n.s. = not significant, *=$p<0.05$, **=$p<0.01$.

2.1.4 MDA-MB-231 cells

In contrast to the cell lines analyzed so far, MDA-MB-231 cells have not been derived from MCF-7 cells. This cell line reveals a consistent cell doubling (about 48h) irrespective of oestrogen or tamoxifen addition (figure 2.11). When day 6 is reached cell numbers have increased by an average of 6.3 fold (control 6.6 fold, E_2

6.5 fold, tam 6.3 fold, E_2 plus tam 5.9 fold). MDA-MB-231 cells proved to be a suitable choice as a control cell line, a cell line that is ERα negative, hormone and antihormone independent and has been established separately from MCF-7 cells.



	C/E_2	C/Tam	C/E_2+Tam	E_2/ E_2+Tam
Day 0	*	n.s.	n.s.	n.s.
Day 2	n.s.	**	n.s.	n.s.
Day 4	*	**	**	**
Day 6	*	n.s.	n.s.	**

Figure 2.11: Cell line characterization of MDA-MB-231 cells. Cells were plated in reduced media for 24h before treatment. Cells were then left untreated (control group), treated with 10^{-9}M E_2; 10^{-6}M tam; or 10^{-9}M E_2 and 10^{-6}M tam. Cells were counted on day 0 (24h after plating/ day of treatment start) and day 2/4/6 using a coulter counter. Cell counts of triplicate samples and duplicate counts for each time point in each treatment group are expressed. Error bars=STD. Representitive experiment is shown of at least two experiments carried out. Table shows significant variance between treatment groups and control group as well as E_2 and E_2+Tam determined by one-way ANOVA and Tukey-Kramer multiple comparison test: n.s. = not significant, *=$p<0.05$, **=$p<0.01$.

2.1.5 Discussion

Human breast cancer cell lines play a vital part in the discovery of mechanisms that lie behind the development of the disease. As with all laboratory models, it is of concern whether cell models represent primary breast cancer tumours. A major review of randomized trials including 37.000 woman with early breast cancer showed that about 53% of the ER-positive tumours did show recurrence after 5 years of treatment (Early Breast Cancer Trialists' Group, 2001). Results therefore indicate that approximately half of ER+ patients are resistant to this form of treatment although such observations have to be interpreted with caution as tumours which did

not recur might also have been tamoxifen resistant but failed to relapse for other reasons. For the metastatic disease, 67% of ER+ patients respond to tamoxifen treatment leaving one third resistant (Dowsett, M. and Howell, A. 2002). Both of these major resistant tumour populations are an example of situations where existing models, here the MCF-7/LCC-1 and LCC-2 cells, are intended to serve as tools to investigate mechanisms of endocrine resistance. Identifying antioestrogen- including tamoxifen- resistant phenotypes is problematic given that adjuvant antioestrogen treatment is often only precautionary and for some patients the disease has been fully eradicated by surgery. But by far the biggest issue in establishing appropriate model systems to accurately reflect clinical situations is the immense heterogeneity of breast tumors. Based on clinical and laboratory observations, there are at least three major ER positive phenotypes with respect to E_2 and antioestrogen sensitivity as suggested by Clarke, R. *et al.* (2001). Firstly, cells may be oestrogen growth dependent, and antioestrogen as well as aromatase inhibitor responsive. Secondly, cells responsive to antioestrogen and potentially aromatase inhibitor might be oestrogen independent but stimulated by the hormone. And lastly, breast cancer cells growing independently of and unresponsive of oestrogen could show crossresistance to different antieoestrogens and aromatase inhibitors. The MCF-7 variant cell line model attempts to cover this range of phenotypes. All lines are ER positive and while the first phenotype is clearly represented by the parental MCF-7 line, MCF-7/LCC-1, LCC-2, LCC-9 and LY2 cells each embody the oestrogen growth independent but differential antioestrogen resistant phenotypes. An example of clinical evidence directly corresponding with an *in vitro* model is the MCF-7/LCC-2 cell line. Published in previous studies, oestrogen independent tumours which had initially been tamoxifen responsive and had subsequently acquired resistance against the agent, showed a significantly higher response rate to ICI182,640 than it would have been expected for the treatment with another similar antioestrogen (Howell, A. *et al.* 1995). This corresponds to the oestrogen unresponsive MCF-7/LCC-2 phenotype that exhibits resistance to tamoxifen but not ICI 182,780.

Aside from distinctive endocrine response features, it is significant how cultured breast cancer cells such as MCF-7 cells compare in their biological properties to the tumours from which they have been derived. Very few comprehensive studies adress

this question. As mentioned earlier, longterm cell culturing can have an impact on hormone sensitivity as a result of which cells have to be tested regularly for their response. Studies examining human breast and lung cancer cell lines side by side with their corresponding tumour tissues determined that, generally, cell lines do retain properties of their original tissues (Wistuba, I.I. *et al.* 1998 and 1999). In the case of 18 breast cancer cell lines, morphologic, phenotypic and genetic changes present in their clinically advanced tumours with poor prognosis correlated amongst others 100% in morphological features, 75% in TP53 gene mutations and 87% in ER protein expression. This suggests that changes in gene expression or cell structure characteristic of advanced metastatic breast cancer are well retained in tissue culture models. Establishing breast cancer tumour cells long-term for culturing is difficult and has only been achieved in a small number of cases (Lacroix, M and Leclercq, G. 2004). However, despite obvious concerns, breast cancer models appear to be representative of their originating clinical specimen.

The MCF-7 mammary tumour cell line has proven to be a particularly useful model system in breast cancer research since its establishment over 30 years ago. MCF-7 cells were isolated from a patient with metastatic breast cancer (Soule, H.D. *et al.* 1973). MCF-7 cells have been shown to proliferate rapidly and are easily maintained *in vitro* and *in vivo*. Most importantly, cells are highly hormone sensitive and multiple receptor positive providing a fundamental model for the analysis of hormone dependent cell growth in breast cancer.

Growth experiments were carried out to confirm basal growth and the effect of oestradiol and tamoxifen on cell proliferation. MCF-7 cells are routinely maintained in phenol red containing media supplemented with 10% fetal calf serum. It is widely acknowledged that phenol red acts as an oestrogen mimic sufficient to stimulate MCF-7 cell growth (Berthois, Y. *et al.* 1986). Also, MCF-7 cells have been shown to adapt to E_2 deprived conditions by increasing the sensitivity to the hormone (Masamura, S. *et al.* 1995). It was therefore essential to remove phenol red and utilize charcoal stripped serum to ensure maximum hormone sensitivity in preparation for all experiments. Results show static MCF-7 cell replication as a result of successful oestrogen depletion (Figure 2.1; control group). In contrast, growth was

markedly stimulated by oestrogen under the same conditions. Tamoxifen acted as an antagonist and partially reversed the stimulatory effect of oestrogen.

A major obstacle in the treatment of breast cancer is that tumors initially oestrogen dependent commonly progress to a hormone independent and drug resistant phenotype. The MCF-7 variant cell lines used in this experiment share a common predecessor and could therefore be used for studies tracing molecular changes during the acquisition of a more malignant phenotype (Brünner, N *et al.* 1993). *In vivo* passaging of MCF-7/MIII cells in overiectomized nude mice resulted in the *in vitro* establishment of the MCF-7/LCC-1 cell line. Cell proliferation experiments showed that LCC-1 cells grow without supplementation of oestrogen. This allows for the cells to be routinely maintained in media supplemented with stripped serum and lacking phenol red. Addition of E_2 to the media does increase proliferation indicating that LCC-1 cells do retain some hormone sensitivity. In contrast, LCC-2 and LCC-9 cells, established by stepwise selection against antioestrogens 4-OH tamoxifen and ICI182,780, respectively, not only proliferated without oestrogen but failed to show any response to it. Both cell lines also demonstrated resistance to tamoxifen. It has previously been shown that while LCC-2 cells retain sensitivity to ICI182,780, LCC-9 cells are characteristic for their antioestrogen cross-resistance (Brünner, N. *et al.*1993, 1997). LCC-2 cells therefore imply that breast cancers resistant to tamoxifen do not necessarily acquire cross - resistance to other anti-hormones simultaneously indicating a situation where one anti-oestrogen treatment option might fail while the other is still effective. LCC-9 cells then provide a phenotype that is cross resistant to different anti oestrogens a situation that proves most difficult for effective treatment

In general, findings regarding hormone and antihormone responsiveness in the MCF-7 and MCF-7 variant lines matched original publications describing their phenotypes. It has been established many years ago that MCF-7 cells are highly oestrogen sensitive after phenol red removal (Berthois, Y. *et al.* 1986). Equally, tamoxifen has been shown to inhibit E_2 stimulated growth (Lippman, M.E. and Bolan, G. 1975). As suggested in the literature, there is no question that all variant lines show the ability to proliferate in the absence of endogenous oestrogen. However, relative hormone sensitivity remains a somewhat elusive issue. For

example, here, LCC-1 cells have shown to be E_2 independent but responsive. It has previously been reported that LCC-1 cells are hormone unresponsive *in vitro* although LCC-1 tumours were stimulated by oestrogen (Brünner, N. *et al.* 1993). Likewise, E_2 has had no mitogenic effect in LCC-2 and LY2 cells in this project but both cell lines have demonstrated oestrogen stimulation, if to a much lesser degree, compared to the parental line (Brünner, N. *et al.* 1997 and Bronzert, D.A. *et al.* 1985). Small differences in hormone response are most likely due to the absence of standardized protocols across the research field. Such interlaboratory variability has been demonstrated for MCF-7 cells (Jones, P.A. *et al.* 1997 among others). Differences in the efficiency in oestrogen stripping of serum or hormone concentrations used in experiments have been shown to have a profound impact on hormone sensitivity and response. With regards to tamoxifen response, LCC-1 cells have been reported to be significantly growth inhibited by tamoxifen. In this study, LCC-1 cells have demonstrated tamoxifen resistance. The published results agree with the results obtained here that LCC-9 and LY2 cells show clear tamoxifen resistance. Since LCC-9 and LY2 cells have been isolated as resistant to ICI 182,780 and LY 117018, respectively, it is perhaps not surprising that resistance against the less potent antioestrogen tamoxifen remains firmly in the phenotype of these cell lines despite potential sensitivity shifts occuring as a result of long-term culturing. ICI 182,640 and LY117018 have a higher affinity for the ER than tamoxifen and may cause more dramatic changes within the ER signalling network (Clarke, R. *et al.* 2001). It has been demonstrated that LCC-9 cells have first become resistant against tamoxifen in the selection against ICI 182,640 (Brünner, N. *et al.* 1997). During routine cell culture and cell maintenance in the absence of antioestrogens, insensitivity to tamoxifen might be the less stable characteristic and revert earlier than the loss of sensitivity to the more potent agent.

The distinct phenotypes with respect to hormone and anti-hormone response mimicking various human breast cancer phenotypes and their common predecessor is what makes these cells a particularly appropriate model system to explore further the mechanistic differences that give rise to these changed growth effects.

2.2 Oestrogen and tamoxifen regulation of oestrogen and progesterone receptor expression

2.2.1 Introduction

Oestrogen is thought to mediate gene expression mainly via ERα. Having established that the MCF-7 variant lines demonstrate different characteristics of growth resistance, it was important to determine oestrogen receptor expression and elucidate whether changes in expression between the MCF-7 parental cell line and its sublines have occurred and might give a first indication of a differential role for the receptor. Another hormone receptor, the progesterone receptor is known to be a primary oestrogen target gene. Differential regulation of PR might provide an insight into ER mediated transcription in these cell lines. First, basal levels of ERα and PR mRNA were determined under oestrogen-deprived conditions. Then, cells were subjected to 10^{-9}M E_2 for 48h and mRNA expression analysed at various time points. Finally, the expression pattern of ERα and PR mRNA and protein in response to 10^{-9}M E_2, 10^{-6}M tamoxifen or E_2 plus tamoxifen were examined at 48h.

The concentrations used for E_2 and tamoxifen as well as the time points chosen for mRNA and protein collection in this experiment have been employed for the entire project. It has long been known that E_2 binding and ER protein turnover can occur in a matter of minutes (Horwitz, K.B. *et al.* 1978; Katzenellenbogen, B.S *et al.* 1987). The effect of E_2 on mRNA and protein expression has been demonstrated to be apparent within both minutes and over a prolonged period of time. For example, Mawson, A. and collegues have recently demonstrated that E_2 induces c-myc protein within two minutes (Mawson, A. *et al.* 2005). The effect is also confirmed at 48h in this study. In addition, the ER has been shown to associate with the promoter of ER mediated genes such as pS2 and CTSD almost immediately after hormone addition (Shang, Y. *et al.* 2000; Metivier, R. *et al.* 2003). My own preliminary results showed that pS2 mRNA expression was altered by the addition of E_2 within hours. Based on this observation, together with the numerous publications, a time course starting as early as 1h but covering a period of 48h was chosen for the experiment.

The utilized concentration of 10^{-9}M E_2 lies well within the range of physiological serum levels of a premenopausal woman considering that levels of freely circulating E_2 in the serum greatly vary around 10^{-9}M to 10^{-10}M during the menstrual cycle of a premenopausal woman (Parl, F.F. (b) 2000). The concentration of 10^{-6}M tamoxifen matches commonly used pharmacological levels in breast cancer therapy (Jordan, V.C. 1982). To demonstrate hormone responsiveness and resistance in the different cell models employed in this thesis, E_2 and tamoxifen concentrations causing maximum stimulatory or inhibitory effects were needed. Dose response curves of E_2 have shown that MCF-7 cells respond maximally at concentrations of 10^{-9}M E_2 and higher (among others: Masamura, S. *et al.* 1995). 10^{-9}M E_2 and 10^{-6}M tamoxifen are commonly used concentration in experiments using hormone sensitive and resistant cell lines observing effects on cell growth but also E_2 mediated gene expression. One example is a study by Chan, C.M.W. *et. al.* (1999). The study analyses the regulation of cofactor mRNA and protein expression in parental MCF- and MCF-7 tamoxifen resistant cell lines comparing expression of cofactors such as RIP140 in the absence and presence of 10^{-9}M E_2 and 10^{-6}M tamoxifen.

2.2.2 ERα and PR mRNA and protein expression in MCF-7 and resistant cell lines

MCF-7 cells and the MCF-7 sublines (LCC-1, LCC-2, LCC-9 and LY2) cells studied expressed ERα mRNA (figure 2.1). The four resistant lines all showed a significantly higher (2-3 fold) level of ERα expression than the parent MCF-7 cells. MDA-MB-231 cells on the other hand did not show any detectable ERα mRNA. Quantitative comparison of ERα mRNA between LCC-1, LCC-2, LCC-9 and LY2 revealed only subtle differences with the level gradually slightly decreasing in the order the cell lines are listed (1.5 fold between LCC-1 and LY2 cells).

PR mRNA presented a different expression profile. MCF-7 and LCC-2 cells expressed particularly low levels of PR mRNA while MDA-MB-231 did not show any expression. Although PR mRNA expression in LCC-1 cells was 4-fold higher than MCF-7, the most markedly different to the parental line was the elevated expression in LCC-9 and LY2 cells (19.6-fold and 15.6-fold, respectively).

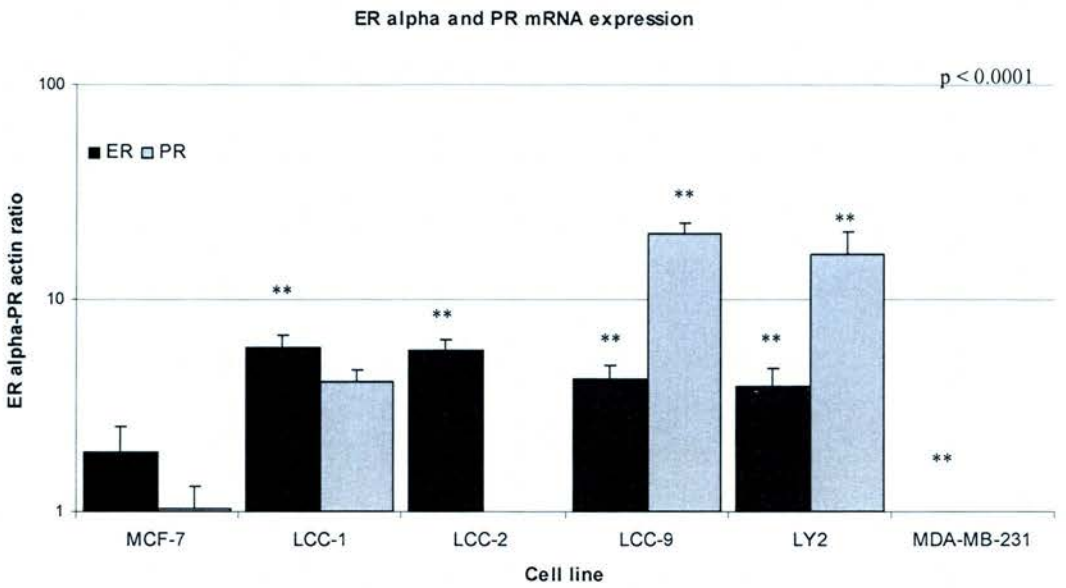


Figure 2.1: ERα and PR mRNA expression in breast cancer cell lines. MCF-7 cells were seeded in complete medium for 24h and a further 48h in reduced media containing 5% DCC. All other cells were seeded in reduced media for 24h before RNA collection. Representative experiment is shown of at least two experiments carried out. Each column presents mean of triplicate PCR analysis for each sample. Error bars = STD. Significant variance between MCF-7 and other cell lines determined by one-way ANOVA and Dunnets multiple comparison test where *=p<0.05, **=p<0.01.

(i) Effect of E_2 on ERα mRNA expression over a period of 48h

As described in the previous section, all cell lines tested contained comparable levels of ERα mRNA with the exception for MDA-MB-231 cells. In MCF-7 cells, ERα mRNA expression was only marginally affected by the addition of E_2 and showed a slight down-regulation at 24h (1.6 fold) (figure 2.2 A and 2.2 B). Down-regulation mediated by E_2 was also observed in LCC-2, LCC-9 and LY2 cells where a gradual reduction led to a maximal 3.6 fold, 2.2 fold and 2.2 fold change, respectively. In contrast, LCC-1 cells revealed an initial up-regulation between 0h and 1h (1.6 fold) before mRNA levels were, again gradually, reduced below baseline levels (1.7 fold at 3h, 1.9 fold at 24h). Any down-regulation was generally found to be at its lowest at 6h or 24h for these cell lines.

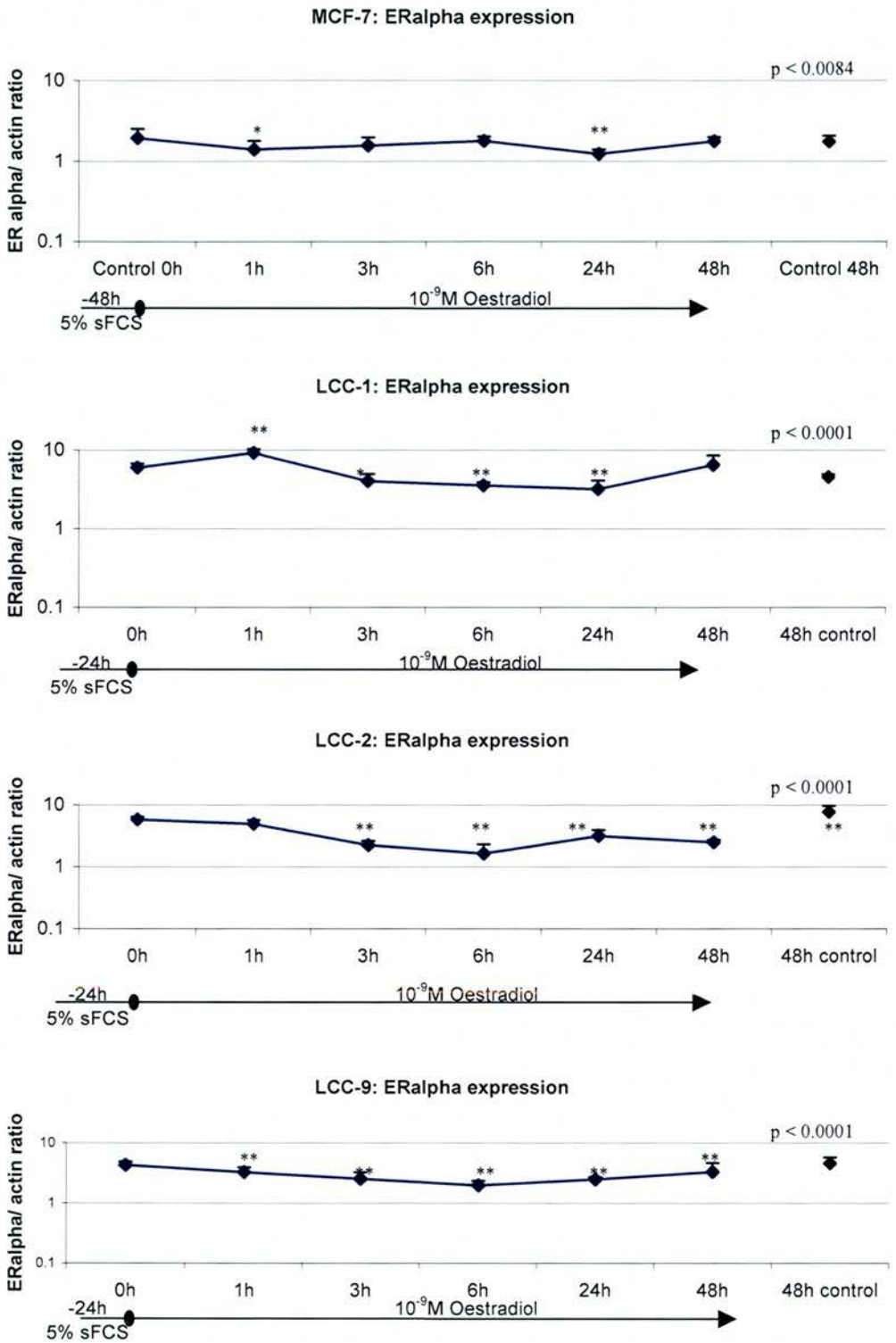


Figure 2.2. A: ERα mRNA expression in MCF-7 wild-type and resistant cell lines. Cells were left untreated (control group) or treated with 10^{-9}M E_2. RNA was collected at 0h, 1h, 3h, 6h, 24h and 48h. A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=p<0.05, **=p<0.01.

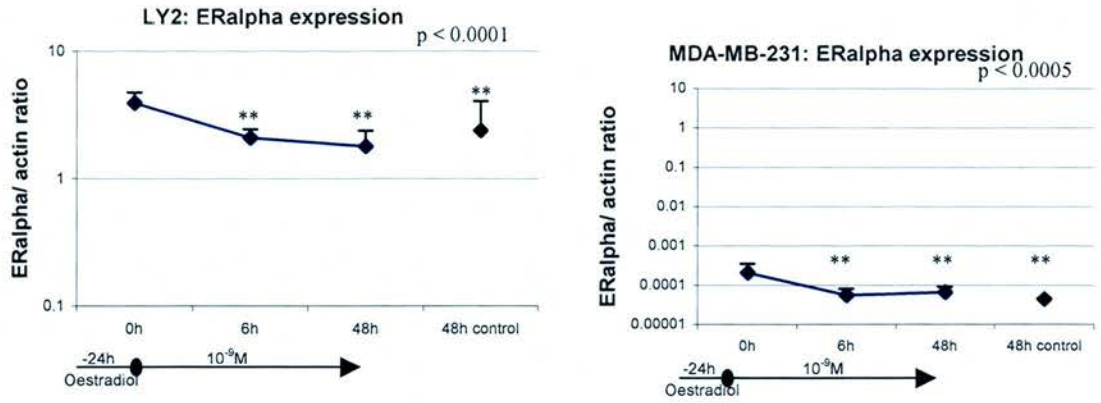


Figure 2.2 B: ERα mRNA expression in LY2 and MDA-MB-231 cells. Cells were left untreated (control group) or treated with 10^{-9}M E_2. RNA was collected at 0h, 6h, 48h. A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=p<0.05, **=p<0.01.

(ii) Effect of E_2 and tamoxifen on ERα mRNA expression at 6h and 48h

Expression studies evaluating ERα mRNA in response to E_2 and tamoxifen distinguished MCF-7 cells from the other breast cancer cell lines (figure 2.3). At 6h as well as 48h, E_2 stimulated ER mRNA expression (2.0 fold and 5.1 fold), an observation that does not match results from the previous E_2 time course. Tamoxifen alone did not have an effect on expression at 6h but was able to suppress the stimulation by E_2 when combined with the hormone.

In LCC-1, LCC-2, LCC-9 and LY-2 cells, observations from the oestrogen time course are confirmed at 6h. E_2 mediated a down-regulation of very similar proportion in all cells (average 2.2 fold). Tamoxifen as well as tamoxifen plus E_2 also decreased expression (average of 2.7 fold and 2.6 fold, respectively). The expression patterns for the MCF-7 variant lines were similar at 48h compared to 6h.

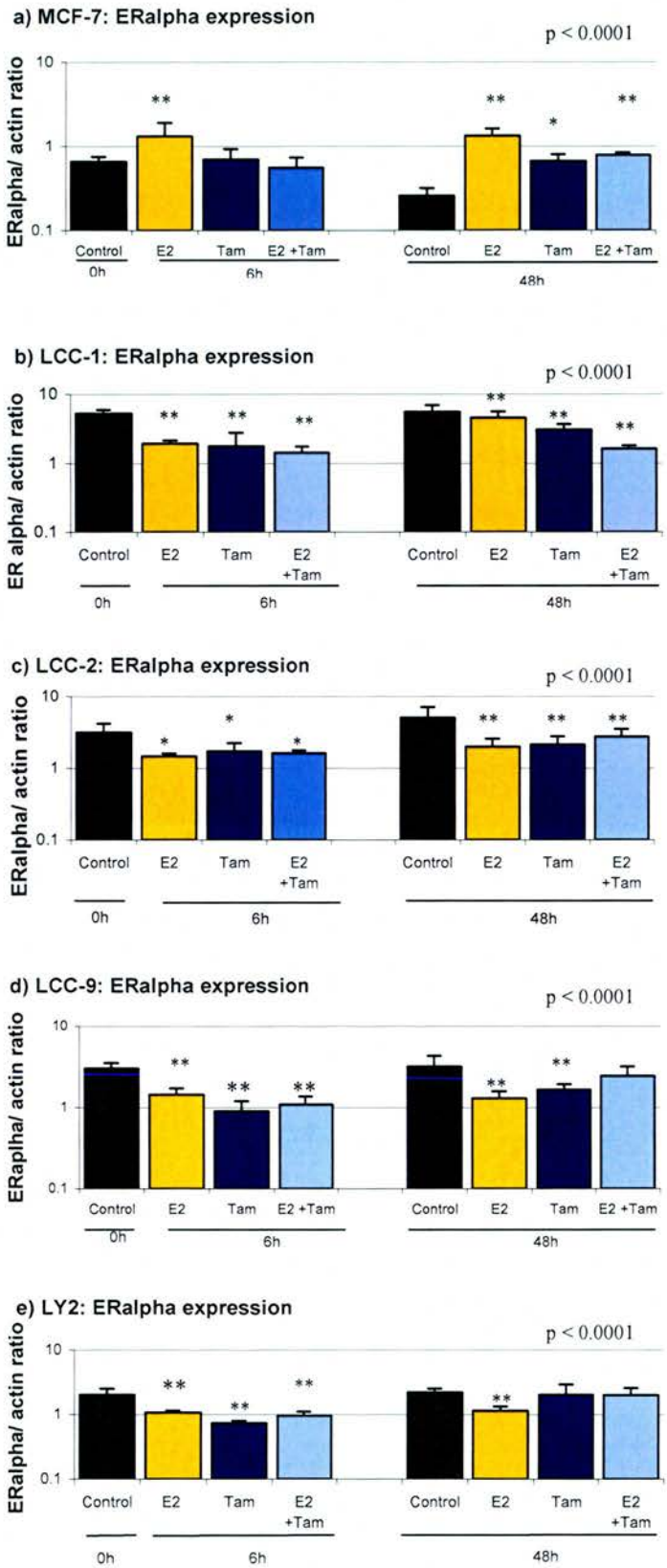


Figure 2.3: ERα mRNA expression in MCF-7, LCC-1/2/9 and LY2 cells. Cells were left untreated (control group), treated with 10^{-9}M E_2; 10^{-6}M tam; or 10^{-9}M E_2 and 10^{-6}M tam. RNA was collected at 6h and 48h. A representative experiment is shown of at least two experiments carried out. Each column presents mean of triplicate PCR analysis for each sample demonstrating mRNA expression relative to actin expression. Error bars=STD. P-value for variance between all groups determined by one-way ANOVA and multiple comparison Tukey test. Statistical significance noted for treatment groups compared to matched control

(iii) Effect of E_2 and tamoxifen on ERα protein expression at 48h

The expression levels of the ERα protein in the cell lines (figure 2.4) corresponded well with the expression of ERα mRNA observed in the previous experiment. ERα protein was present in all MCF-7 variants but not in MDA-MB-231 cells. 1-D band analysis providing approximate OD values confirmed that the basal levels of ERα protein are strongest in LCC-1 and LCC-2 cells and less strong in LCC-9 and LY2 cells (table 2.2). MCF-7 cells showed ERα expression levels comparable to LY2 cells. The expression difference between MCF-7 and LCC-1/ LCC-2 was prominent.

ERα protein expression was significantly decreased by E_2 in all ER positive cell lines. Tamoxifen and the combination of E_2 plus tamoxifen had a stimulated ERα protein expression in MCF-7 and LCC-1 cells. In LCC-2 cells, ERα protein expression in the presence of tamoxifen was similar to basal levels but this was markedly reduced by the addition of E_2. Remarkably, the presence of E_2 and/ or tamoxifen produced undetectable levels of ERα protein in LCC-9 and LY2 cells.

Taken together, the observations indicate characteristic ERα protein expression patterns in response to E_2 and tamoxifen for MCF-7 cells. Unique protein expression has been shown for each of the variant lines. Oestrogen decreased ERα expression in MCF-7 and all variant cell lines. Novel observations have been made with protein expression in the presence of tamoxifen in variant lines. Unlike in MCF-7 cells, tamoxifen alone led to no change in LCC-1 cells and reduced expression in LCC-9 and LY2 cells. In combination with E_2, tamoxifen significantly reduced protein expression in LCC-2, LCC-9 and LY2 cells.

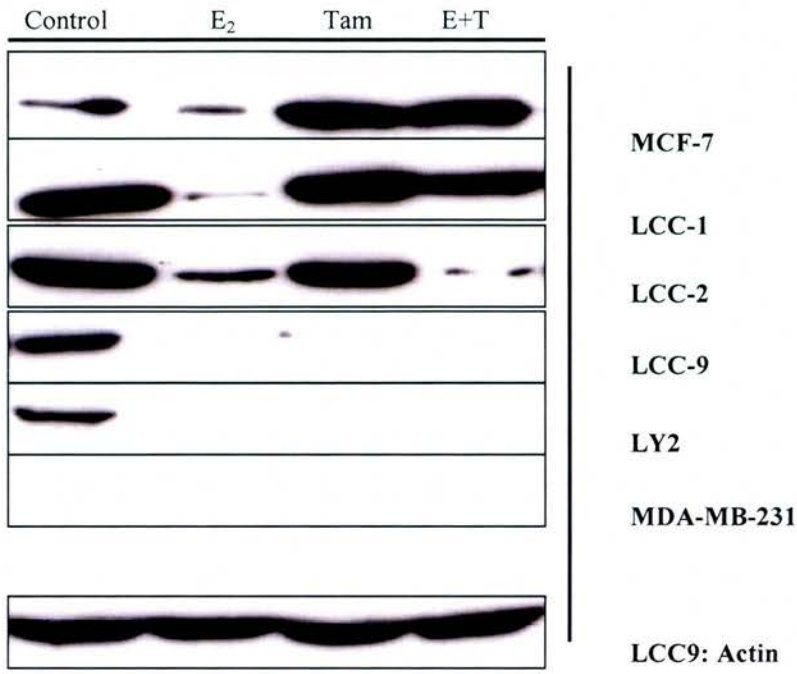


Figure 2.4: Western blot analysis of ERα (66kDa) in breast cancer cell lines in control, 10^{-9}M E_2, 10^{-6}M tam or 10^{-9}M E_2 and 10^{-6}M tam treated groups at 48h. 100µg protein was loaded per lane and detected using anti-ERα antibody (Santa Cruz Biotech). Total actin (42kDa) was detected using anti – β-actin (CALBIOCHEM®) in all cell lines (one representative cell line shown) as a western blot loading control.

Sum OD by 1D band analysis				
	Control	**Oestrogen**	**Tam**	**E+Tam**
MCF-7	382.5	137.8	1677.4	1118.4
LCC1	2553.3	25.5	1565.7	1804.4
LCC2	1617.3	445.0	1893.2	101.72
LCC9	822.83	nd	14.81	nd
LY2	426.62	nd	nd	nd
MDA	nd	nd	nd	nd
Actin				
LCC2	1745.50	1668.10	1349.80	1157.70
LCC9	1987.60	1907.60	1425.70	1480.10

Table 2.2: Approximate total OD values of ERα protein (67kDa) obtained from Western blot (Figure 2.4) using LabWorks3.0 and 1D band analysis.(nd=not detected).

2.2.3 PR mRNA and protein expression in MCF-7 and resistant cell lines

(i) Effect of E_2 on PR mRNA expression over a period of 48h

MCF-7 and its variant cell lines, LCC-1, LCC-2, LCC-9 and LY2, demonstrated expression of progesterone receptor mRNA (figure 2.5 A and B). Oestrogen addition significantly up-regulated the expression, most profoundly in MCF-7 cells (32.7 fold) and least of all in LCC-9 cells (1.8 fold). The up-regulation was gradual and continuous up to 48h. However, the effect of E_2 was not immediate. In MCF-7 and LCC-1 lines an increase in PR mRNA was revealed only after 3h. A further time delay was observed in other cell lines: in LCC-2 cells after 6h and in LCC-9 cells after 24h. MDA-MB-231 cells show extremely low levels of PR mRNA expression.

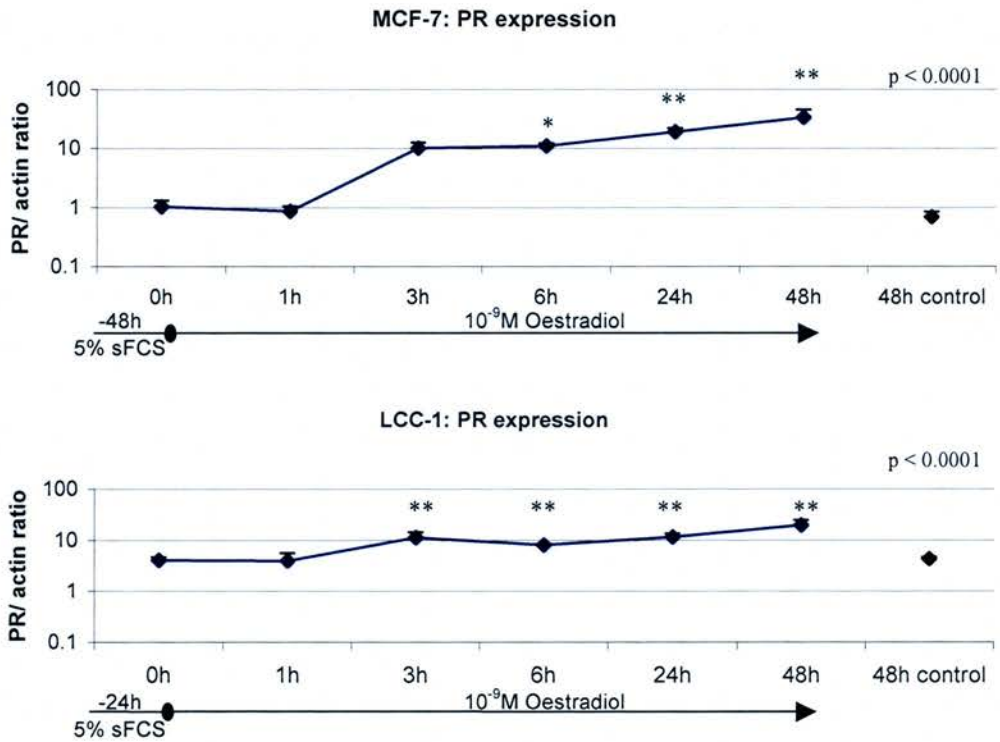


Figure 2.5 A: PR mRNA expression in MCF-7 and LCC-1 cells. Cells were left untreated (control group) or treated with 10^{-9}M E_2. RNA was collected at 0h, 1h, 3h, 6h, 24h and 48h. A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=$p<0.05$, **=$p<0.01$.

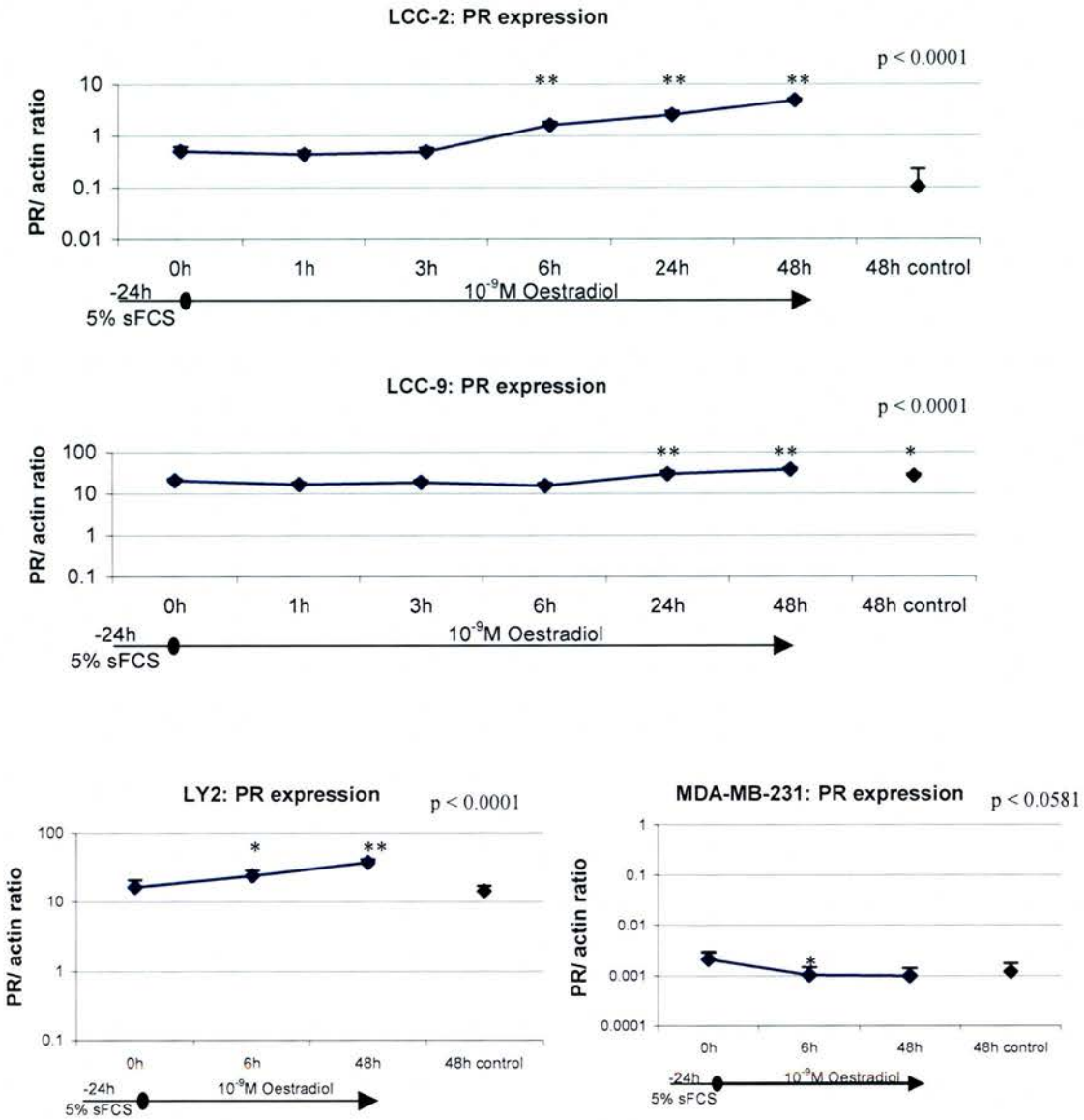


Figure 2.5 B: PR mRNA expression in MCF-7, LCC-1/2/9 and LY2 cells. Cells were left untreated (control group) or treated with 10^{-9}M E_2. RNA was collected at 0h, 1h, 3h, 6h, 24h and 48h (0h, 6h, 48h only for LY2 and MDA-MB-231 cells). A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=p<0.05, **=p<0.01.

(ii) Effect of E_2 and tamoxifen on PR mRNA expression at 6h and 48h

The effects of oestrogen and tamoxifen in these cell lines were next evaluated at 6h and 48h (figure 2.6). The E_2 stimulated up-regulation of the PR mRNA in all analysed breast cancer cell lines with the exception of MDA-MB-231 cells was again observed in this experiment. Most marked are the effects when comparing 0h to 48h reflecting the continuous stimulation of the hormone. In general, tamoxifen and E_2 plus tamoxifen only showed significant regulatory effects at 48h.

In MCF-7 cells, E_2 stimulated PR mRNA at 6h (50 fold) and most dramatically at 48h (1017.1 fold). Tamoxifen and the combination of tamoxifen and E_2 cause small increases in mRNA expression at 48h. In LCC-1, LCC-2, LCC-9 and LY2 cells, E_2 induced mRNA expression slightly at 6h but did not reach statistical significance. At 48h, E_2 inducbility is more obvious reflecting the increasing time delay seen in the previous experiment, the oestrogen time course. Again, a much smaller stimulation is observed in LCC-9 cells (1.7 fold) compared to LCC-1 cells (11.3 fold) and LCC-2 cells (8.7 fold). Tamoxifen alone and tamoxifen plus E_2 showed stimulatory effects at 48h in LCC-1and LCC-9 (tam 8.1 fold and 1.6 fold, E_2 plus tam 11.5 fold and 2 fold, respectively) comparable to the effects observed with E_2 in those cells.

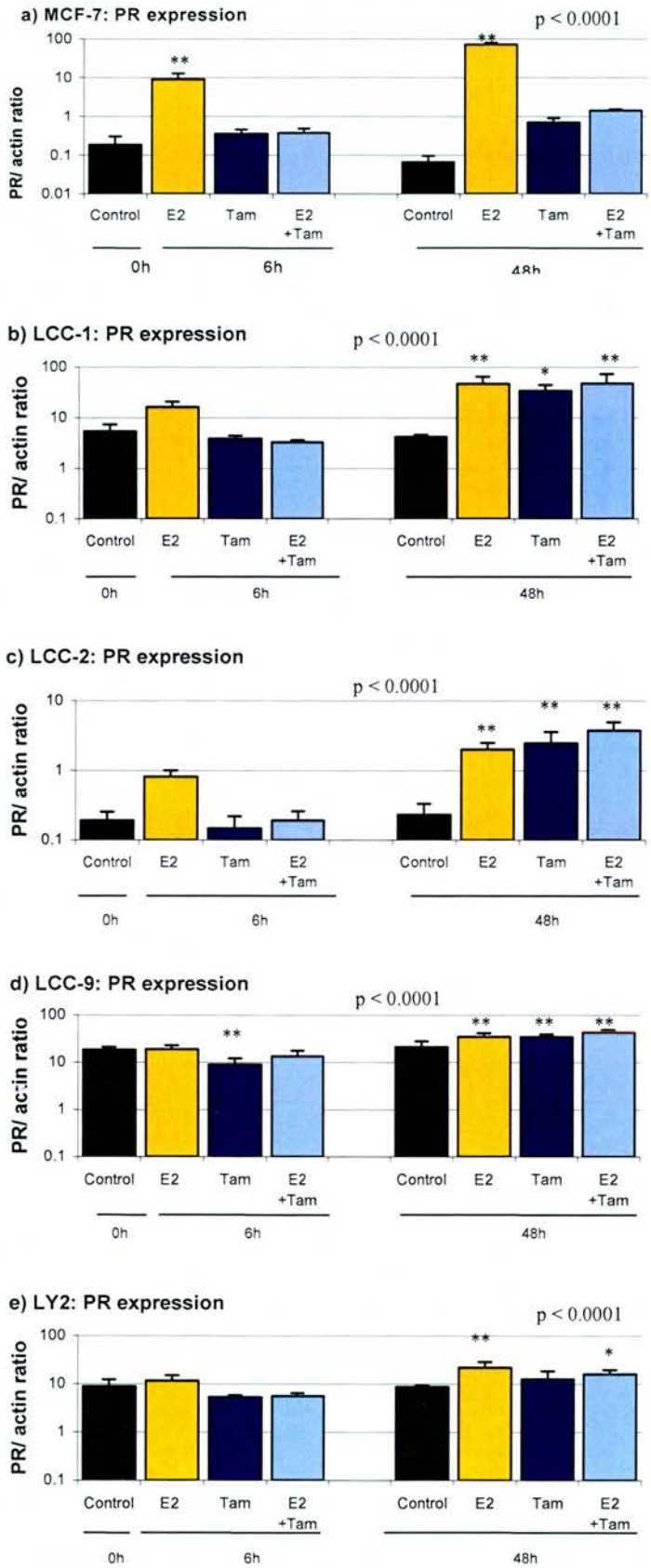


Figure 2.6: PR mRNA expression in MCF-7, LCC-1/2/9 and LY2 cells. Cells were left untreated (control group), treated with 10^{-9}M E_2; 10^{-6}M tam; or 10^{-9}M E_2 and 10^{-6}M tam. RNA was collected at 6h and 48h. A representative experiment is shown of at least two experiments carried out. Each column presents mean of triplicate PCR analysis for each sample demonstrating mRNA expression relative to actin expression. Error bars=STD. P-value for variance between all groups determined by one-way ANOVA and multiple comparison Tukey test. Statistical significance noted for treatment groups compared to matched control where *=$p<0.05$, **=$p<0.01$.

(iii) Effect of E_2 and tamoxifen on PR protein expression at 48h

In MCF-7 cells, progesterone receptor protein could not be detected in the absence of E_2 (figure 2.7). The addition of E_2 resulted in the presence of a strong band with a size of about 116kDa representing protein of isoform hPR-B and about 81kDA for the isoform hPR-A. PR protein was also strongly up-regulated by the presence of E_2 plus tamoxifen. Tamoxifen alone only marginally increased PR protein expression compared to the untreated control. Protein expression broadly follows the expression pattern observed at the transcriptional level.

LCC-1 and LCC-2 cells also demonstrated E_2 up-regulated PR protein (approximately 11.1 fold and 24.4 fold, respectively). PR protein expression patterns with and without oestrogen are comparable between MCF-7 cells and LCC-1/LCC-2 cells. The hormone inducibility has also been demonstrated at PR mRNA expression. While tamoxifen alone stimulates PR protein expression in LCC-1 cells, it has no effect in LCC-2 cells where expression was low and comparable to basal levels. Tamoxifen does not reduce E_2 stimulation in either of the cell lines when combined with the hormone. Interestingly, as observed for mRNA expression, in LCC-9 cells PR protein expression was constitutively high. The receptor showed marginal oestrogen inducibility (approximately 1.7 fold). PR protein expression in LY2 was low and undetectable in MDA-MB-231 cells. This stands in contrast to strong PR mRNA expression in LY2 cells. Both isoforms of the progesterone receptor were present in the PR positive cell lines with a predominant expression of hPR-B.

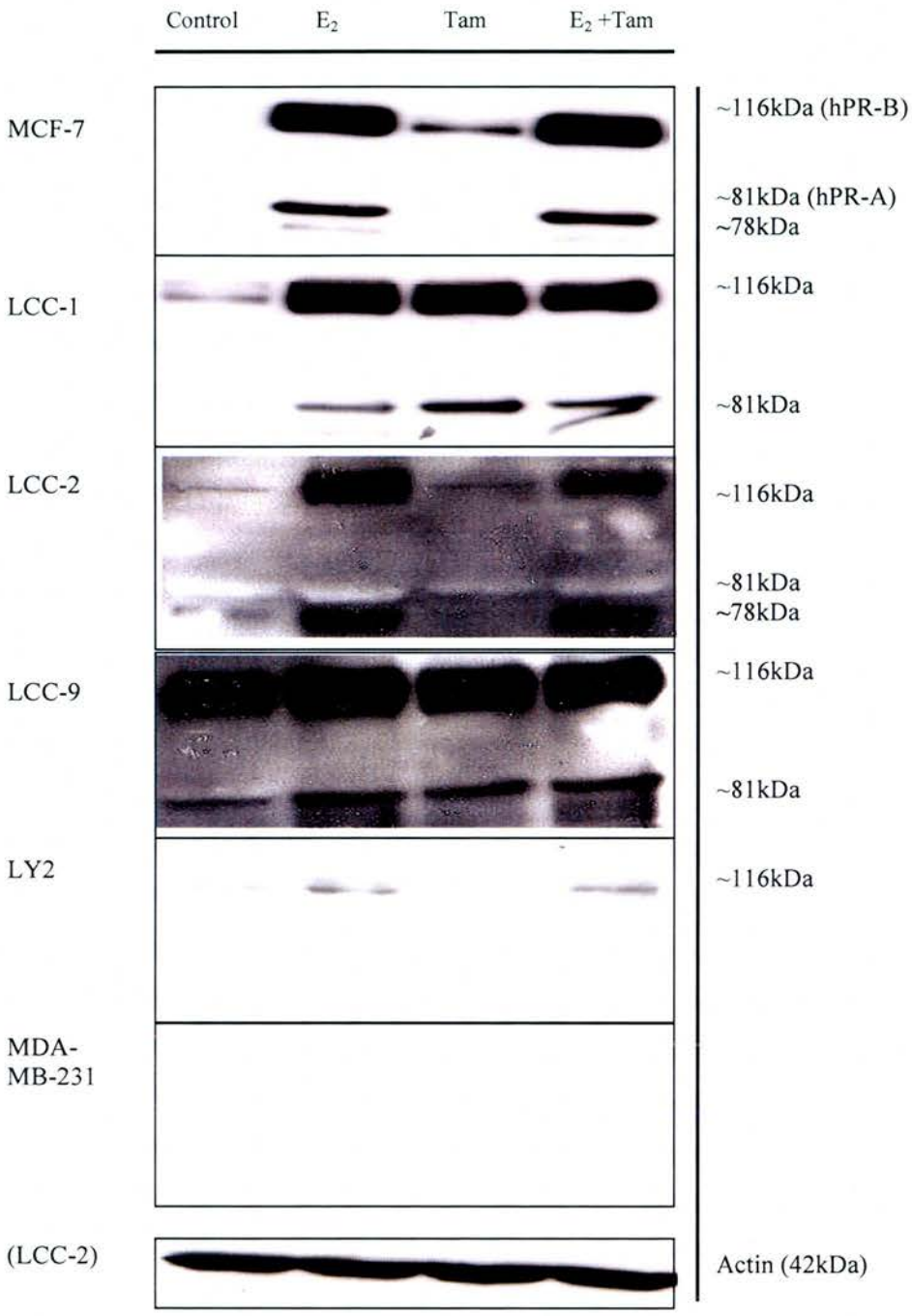


Figure 2.7: Western blot analysis of PR in breast cancer cell lines in control, 10^{-9}M E_2, 10^{-6}M tam or 10^{-9}M E_2 and 10^{-6}M tam treatments at 48h. 100μg protein was loaded per lane and detected using anti-PR antibody (Neomarker). Total actin was detected using anti - β-actin (CALBIOCHEM®) in all cell lines (one representative cell line shown) as a western blot loading control.

Sum OD by 1D band analysis					
PR	Band Size(kDa)	Control	Oestrogen	Tam	E+Tam
MCF-7	116	nd	2914.2	580.7	3252.2
	81	nd	1113.5	nd	1177.5
	79	nd	66.0	nd	27.7
LCC1	116	190.0	2103.2	1890.9	3061.3
	81	nd	234.1	723.2	657.3
	79	nd	37.1	nd	nd
LCC2	116	64.5	1576.6	354.3	1457.3
	81	nd	1397.2	nd	1666.5
	79	nd	nd	nd	nd
LCC9	116	3409.6	5866.7	2379.4	4295.9
	81	375.4	1420.4	1060.5	967.4
	79	421.2	897.9	610.11	501.3
LY2	116	29.6	22.8	nd	122.4
	81	nd	nd	nd	nd
	79	nd	nd	nd	nd
MDA	-	nd	nd	nd	nd
Actin					
LCC2	42	1316.4	1471	1730.5	1684.4

Table 2.3: Absolute OD values of PR protein obtained from Western blot (Figure 2.7) using Labworks and 1D band analysis (nd = not detected).

In summary, PR protein was generally E_2 inducible in MCF-7 and MCF-7 variant cells. Patterns of expression were comparable in MCF-7, LCC-1 and LCC-2 cells with the exception of tamoxifen treated LCC-1 cells. Consistently highest PR protein expression was found in LCC-9 cells. Both, LY2 and MDA-MB-231 cell express insignificant or undetectable amounts of progesterone receptor protein. Protein expression does not always mirror mRNA expression indicating that rates of turnover as well as synthesis impact in the levels of expression.

2.2.4 Discussion

ERα plays a key role in the development of the normal breast and has been shown to participate in the progression of breast cancer. The receptor serves as a means for its ligand to mediate most of its known effects and study of the receptor lies at the core of this project. The ER ligand binding domain not only binds E_2 but directly associates with transcriptional coactivators and corepressors (Enmark, F. and Gustafsson, J.-Å. 1999). Another target of oestrogen is the progesterone receptor. Its role in breast cancer development remains poorly understood but its functions in endocrine signalling are thought to be mainly due to its ability to oppose transcriptional activity of the ERα. The PR isoform hPR-A is known to antagonize the transcriptional activity of ERα in particular (Giangrande, P.H. and McDonnell, D.P. 1999). E_2 itself increases PR expression (Graham, J.D. and Clarke, C.L. 1997). This serves as evidence for the complex linking of the receptor pathways particularly since the progesterone receptor is unable to heterodimerize with the oestrogen receptor. It is crucial to identify the differential role ER may play in endocrine resistance by comparing expression pattern as well as response to oestrogen and antioestrogens in resistant and non-resistant breast cancer cell lines. Insight into the complex relationship of ER and PR might be beneficial in gaining this knowledge.

It is well documented that MCF-7 cells not only express ERα but that the expression is influenced by oestrogen and tamoxifen (Horwitz, K.B. and McGuire, W.L. 1978 (a) and Eckert, R.L. *et al.* 1984). These observations are confirmed here. A significant down-regulation of ERα protein was observed after long-term E_2 addition (48h) while both tamoxifen and tamoxifen plus oestrogen addition result in up-regulated ER protein expression. The effect of oestrogen on ERα over the course of 48h was marginal but contrasted with the second experiment where a slight increase was observed at 6h and 48h. These conflicting results might partially be due to shifting baseline expressions at 0h and 48h. Published studies suggest that ERα mRNA is down regulated by E_2 after 6h and up to 48h in MCF-7 cells (Saceda, M. *et al.* 1988; Cho, H.S. *et al.* 1991). Decreased ERα protein expression indicates an increased ERα use in the presence of its ligand, supported by steady mRNA levels, coupled with a rapid turnover observed in decreased protein expression long-term.

Early work on ERα has established that hormone binding takes place within 5min and a rapid decrease of ERα protein levels occurs after 30min (Horwitz, K.B. and McGuire, G.L. 1978(a); Katzenellenbogen, B.S. *et al.* 1987). This processing continues for 3-5h and remains at a low level for the remainder of the oestrogen exposure. In fact, ERα half-life has been established to be approximately 3-4h (Monsma, F.J., Jr. *et al.* 1984). The short-term unaffected but long-term slightly increased ERα mRNA expression after tamoxifen treatment matched with increased protein levels at 48h may confirm the much lower receptor turnover rate with the antioestrogen (Horwitz, K.B. and McGuire, W.L. 1978 (a)). If tamoxifen competes with oestrogen for the binding sites, the tamoxifen-bound receptor is no longer processed at the rapid rates seen with E_2. As the receptor becomes processed at a much lower rate, protein is allowed to accumulate.

All the MCF-7 variant cell lines studied express ERα mRNA and ERα protein. Baseline levels of ERα mRNA vary only marginally between the variant lines but are generally slightly higher than in MCF-7 cells. This may be due to the resistant cells not being exposed to E_2 in passaging in charcoal stripped serum conditions. The researchers who established the LCC series of cell lines observed 'essentially comparable ER expression levels' in these lines (Brünner, N. *et al.* 1997). The fact that ERα gene and protein are expressed at comparable or higher levels in the E_2 resistant cell lines obviously eliminates the theory that loss of the receptor is reponsible for the change in phenotype. In fact, in a tet-inducible ERα overexpressing MCF-7 system, it has been demonstrated that high concentrations of the receptor can stimulate gene expression of E_2 target genes such as pS2 in a hormone independent manner (Fowler, A.M. *et al.* 2004). This clearly indicates that a mechanism comes into place where the receptor is activated in the absence of E_2 and continues to mediate gene transcription in these phenotypes. This is discussed further in subsequent chapters.

Interestingly, both E_2 and tamoxifen treatment lead to reduced ERα mRNA expression in all resistant lines. The addition of oestrogen revealed a uniform protein down-regulation leading to non-detectable levels of ERα in LCC-9 and LY2 cells. Tamoxifen only reverses this effect in MCF-7, LCC-1 and LCC-2 cells. Decreased

protein levels in response to oestrogen correspond to ERα mRNA expression pattern in all of the MCF-7 variants while tamoxifen and tamoxifen plus oestrogen effect on protein varies between protein and mRNA expression. The absence of the ERα in MDA-MB-231 cells was confirmed (Cailleau, R. *et al.* 1978). The fact that cells responding differently to oestrogens and antieostrogens with respect to growth all retain the ERα is what makes this cell model particularly interesting for the study of endocrine resistance mechanism. This verifies that loss of the oestrogen receptor is not the underlying cause of endocrine resistance in these models. In addition, the observed endocrine independence but responsiveness observed in some cells points out that the receptor might still be functional although it is likely that receptor signalling has undergone dramatic changes.

The main focus of research in these cell lines surrounds the functionality of the receptor. There is no doubt that a selective process comes into place where cells slowly adapt to grow without hormone supplementation. The identification of several oestrogen mimicking chemicals in the laboratory environment such as alkyl phenols released from plastic centrifuge tubes (Soto, A.M. *et al.* 1991) might suggest that minimal levels of growth hormone is available for cell growth adapted to such small concentrations. A stepwise selection to oestrogen independence cells could also involve various mutations of the oestrogen receptor. Mutations might result in the receptors altered ability to bind oestrogen and/or antioestrogen, to associate with the promoter of the target gene or interact with transcription factors. Several mutated forms of the receptor have been detected. A constitutively active receptor is the result of the amino acid Tyr^{537} substitution by Ala, Ser, Asp, or Glu (Weis, K.E. *et al.* 1997; Zhang, Q.X. *et al.* 1997; Sommer, S and Fuqua, S. 2001). This mutant has shown a conformational shift in the LBD and is also able to bind coactivators such as SRC-1 in the absence of the ligand. An identified shorter ERα isoform hER-α46 is thought to be involved in cell proliferation in MCF-7 cells (Flouriot, G. *et al.* 2000). An increasing number of ERα mRNA splice variants have been identified *in vitro* and *in vivo.* In MCF-7 and endocrine resistant MCF-7 variant lines with resistance to tamoxifen, similar expression levels of ERα splice variants lacking exon3 to exon 5 and exon 7 were demonstrated but corresponding proteins could not be identified

(Madsen, M.W. *et al.* 1997). Other groups have identified distinct expression patterns of ERα wild type and ERα splice variants in normal and malignant breast cancer cells including the MCF-7/LCC1/2/9 sublines (Poola, I. *et al.* 2000 and 2001). Published results and speculations vary with respect to presence and possible involvement of mutant receptors in breast cancer. However, it is unlikely that this is the underlying cause for the development of endocrine resistance. However, the expression of the receptor across different endocrine resistant phenotypes and the varying response to endocrine agents despite apparent endocrine independent growth demonstrates its continued role in oestrogen pathways. It is possible that tumour specific receptor expression patterns including mutants might contribute to the adaptability of the tissue to respond to endocrine agents.

It becomes evident that if oestrogen and antioestrogen binding does differentially mediate ERα expression at the mRNA and protein level, alternative pathways must be initiated upon ligand binding. Oestrogens as well as tamoxifen down-regulate ERα mRNA in all MCF-7 variant cell lines. ERα protein is generally down-regulated in these cell lines and not detectable in the presence of E_2 and tamoxifen in the antioestrogen cross-resistant MCF-7/LCC-9 and LY2 cells. In MCF-7 cells, ERα protein levels are increased upon the addition of tamoxifen. Much thought has been given to evidence suggesting use of alternate promoters within the ERα gene for the transcriptional regulation of the receptor (MacGregor and Jordan, V.C. *et al.* 1998). Additionally, several regulatory elements upstream of the transcription start site have been identified indicating the possibility of differentially influencing ER transcription regulation. The small ER-EH0 enhancer element has been shown to be active in ER+ but not ER- breast cancer cells and specific DNA protein complexes associate with this element (Tang, Z. *et al.* 1997). Use of alternate promoters could be used in response to changed environments within the cell such as the continued presence of an antioestrogen. Ligand binding and interaction of the receptor with target genes or their transcription complex can also be facilitated by receptor phosphorylation of specific serine or possibly tyrosine residues (Nilsson, S. *et al.* 2001). MAP kinase activated phosphorylation of Ser^{118} within the N-terminal region of the oestrogen receptor is thought to enhance ERα AF-1 activity thereby stimulating protein-protein interactions to increase gene transcription (Kato, S. *et al.*

2000). Cross-signalling between the ER and other growth factors is most often linked to a common mediator, the MAPK cascade. Regulation becomes a complex network as MAPK activation in turn is influenced by growth factors such as IGF, insulin and TGF-α.

Cross-talk between the oestrogen receptor and other signalling pathways also leaves open the possibility of ligand independent receptor activation. Again, this corresponds to breast cancer tissue modelled by MCF-7/LCC-1 or LCC-2 cells that grow independently of oestrogen but are sensitive to oestrogen or an antioestrogen (Clarke, R. *et al.* 2001). EGF, TGFα, dopamine or heregulin have all been have shown to indirectly activate the oestrogen receptor via crosstalk to the respective signalling pathways (Gruber, C.J. *et al.* 2002). For example, overexpression of HER-2 can lead to oestrogen and tamoxifen independent cell growth and HER-2 activation by its ligand heregulin leads to ER activation via phosphorylation indicating a potential involvement for this ER ligand independent pathway in the development of endocrine resistance (Pietras, R.J. *et al.* 1995).

Ligand independent pathways to alternatively regulate transcription involving intracellular kinase activity have also been suggested for other nuclear receptors such as progesterone receptor activation. This may also involve complex cross-signalling between signalling pathways and provide possible mechanisms to develop antioestrogen resistance. The protein kinase (PKA) stimulator cyclic AMP (cAMP) has been shown to activate the progesterone receptor in the absence of its ligand (Denner, L.A. *et al.* 1990). Further, PR stimulatory effects observed for oestrogen have been demonstrated to be similar in magnitude for cAMP or IGF-1 linking progesterone signalling not only with the oestrogen receptor but with the protein kinase and insulin receptor pathways, respectively (Aronica, S.M. and Katzenellenbogen, B.S. 1991). Respective antagonists of the three agents such as the antieostrogen ICI 164,384 for E_2, reversed this stimulation. Effects of E_2, IGF and cAMP were not additive suggesting a complex network with a common element such as MAPK signalling as a central mediator (Aronica, S.M. and Katzenellenbogen, B.S. 1991).

MCF-7 cells were shown to express low levels of PR mRNA in the absence of oestrogen. Protein expression could not be detected under these conditions. This

might partially be based on the superior sensitivity of RT-PCR relative to Western blotting although not all mRNA copies are necessarily translated into protein. Processing of mRNA might prevent protein expression. Most interestingly, oestrogen strongly increased mRNA expression in a progressive manner over the course of 48h by which time protein was also strongly expressed. This stimulatory effect was generally also observed for the expression of PR mRNA and protein in MCF-7 variant lines although inducibility was clearly strongest in MCF-7 cells. Tamoxifen acted as an agonist inducing mRNA and protein expression long-term across the panel of cell lines. Overall PR protein expression was comparable between MCF-7 and LCC1/2 cells, slightly higher in LCC-9 but lower in LY2 cells. Small differences of PR protein expression between MCF-7 and LCC lines had been published previously confirming a higher PR protein content in LCC-1 and LCC-9 cells, and a somewhat lower level in LCC-2 cells (Brünner, N. *et al.* 1993 and 1997). Upon establishment, LY2 cells had been reported as PR negative with and without oestrogen (Bronzert, D.A. *et al.* 1985). The presence of oestrogen inducible PR mRNA and protein in this experiment might indicate the availability of better quality antibodies now available and the resulting possibility of distinguishing between the different isoforms of the receptor at the protein level.

The constitutive and long-term stimulation of PR mRNA and protein expression in the presence of oestrogen is likely to be based on the cross-signalling between the oestrogen and the progesterone receptor and their role in normal mammary gland development. Expression of the progesterone receptor changes from a more uniform to a clustered pattern during distinct phases of female reproductive system development such as puberty and pregnancy (Mulac-Jericevic and Conneely, O.M. 2004; Anderson, E. 2002). ERα or PR expressing cells and cells actively dividing cells have been suggested to be two separate populations since, in normal breast epithelium, cells containing steroid receptors have shown to be expressed separately but adjacent to actively proliferating cells (Clarke, R.B. *et al.* 1997; Russo, J. *et al.* 1999). It is therefore assumed that progesterone regulates cell proliferation in an indirect manner by signalling the release of growth factors only after prolonged oestrogen exposure. The continuous presence and accumulation of oestrogen in this experiment resembles this situation and leads to an increased expression of the

progesterone receptor. During carcinogenesis, a change in growth factor signalling might also mimic a constitutive hormone presence and lead to uncontrolled cell proliferation and the development of malignant breast tissue. Interestingly, in breast tumours, about 65% showed a larger number of proliferative cells expressed ER supporting altered cell signalling and gene expression (Clarke, R.B. *et al.* 1997).

Perhaps most intriguing is the question of whether the balance between the two progesterone isoforms could explain differing endocrine response in breast cancer tissue. Somewhat conflicting evidence has been collected since the isolation of the isoforms in the 1980s. In normal mammary tissue, the expression of hPR-A and hPR-B is thought to be close to even (Mote, P.A. *et al.* 2002). A predominant expression of one form or the other has been reported in some publications (Graham, J.D. *et al.* 1996 or Mote, P.A. *et al.* 2002). Here, the predominantly expressed form was hPR-B in all cell lines. An additional third and smaller band of about 75kDA was also often found. Other investigators have reported this finding (Graham, J.D. *et al.* 1996). The main hPR-A and hPR-B isoforms have been assigned distinct functionality. While hPR-B acts as a transcriptional activator, h-PR-A might act predominantly as a repressor and mask the actions of hPR-B where isoforms are co-expressed (Mulac-Jericevic and Conneely, O.M. 2004). This might indicate a poor response to endocrine treatment in cells were hPR-A is predominantly expressed particularly because this isoform has shown to repress other growth factor pathways such as the oestrogen receptor. But there is also evidence that isoform hPR-B is capable of repressing transcriptional activity in line with observations made here in MCF-7 and MCF-7 variant lines. Oestrogen dependent activation of pS2 was repressed only in modified MDA-MB-231 cells designed to selectively oeverexpress hPR-B and ERα (Chalbos, D. and Galtier, F. 1994). This result was also true for hPR-B overexpressing MCF-7 or HeLa cells and could not be demonstrated for hPR-A constructs.

The potential functionality difference between isoforms is underlined by the identification of an additional activation domain in hPR-B (Graham, J.D. and Clarke, C.L. 2002). The AF-3 region, like the common AF-1 sequence, has been shown to recruit coactivators. This leaves the possibility that the isoforms not only attract unique sets of transcriptional cofactors but also show differential binding affinities

for the same promoters to mediate gene transcription. Isoforms have been demonstrated to express specific peptide binding affinities (Giangrande, P.H. *et al.* 2000). Microarray studies have revealed progesterone mediated promoter specific transcription regulation in T47D breast cancer cells (Richer, J.K. *et al.* 2002). When hPR-A or hPR-B were expressed exclusively unique sets of genes mediated by each isoform were identified with only a small percentage of overlap. Interestingly, when isoforms were co-expressed in the wild type, regulation was found to be much less restricted. Similar to the observation where hPR-A is able to reverse transcriptional activity of hPR-B, hPR-B might be able to mask repressive functions of hPR-A and utilise its additional region AF-3 to enhance transcriptional activation. The predominant expression of hPR-A or hPR-B might therefore lead to promoter and tissue specific PR mediated gene transcription. A change in PR isoform expression ratio between normal and malignant breast tissue might lead to differential endocrine responsive phenotypes.

2.3 Regulation of ER target genes by oestrogen and tamoxifen

2.3.1 Introduction

The oestrogen receptor classically mediates transcription by direct contact with oestrogen response elements (EREs) or alternative response elements on the target gene. Cathepsin D (CTSD), pS2 and MYC are three genes whose trancription is well known to be E_2 mediated (Cavailles, V. *et al.* 1989; Dubik, D. *et al.* 1987). All have previously been identified in breast cancer tissues (Rochefort, H. *et al.* 1987; Masiakowski, P.*et al.* 1982; Escot, C. *et al.* 1986) To measure the expression of pS2, CTSD and MYC mRNA in MCF-7, MCF-7 variant and MDA-MB-231 breast cancer cell lines a similar series of experiments to the ones described in the previous section were undertaken. RNA was prepared from cultured cells and amplified using the RT-PCR technique. Results are expressed as actin ratios and statistically analysed as described previously.

2.3.2 MCF-7 and MCF-7 variant cells show divergent expression of pS2, CTSD and MYC mRNA

Basal levels of pS2 mRNA were detected in all but the ER negative MDA-MB-231 cells (Figure 2.8). The parental MCF-7 cell line presents a very low level of pS2 mRNA under these oestrogen deprived conditions compared to LCC-1, LCC-2 and LCC-9 cells which express elevated almost identical levels (66 fold, 65 fold and 73 fold, respectively relative to MCF-7 cells). In contrast, CTSD as well as MYC mRNA expression were revealed to be relatively low for all cell lines. Differences in expression for both genes between cell lines were not as dramatic. One noteworthy exception is the high expression of CTSD in MDA-MB-231 cells compared to MCF-7 cells (4.8 fold). Expression of MYC mRNA is generally higher in LCC-1/2/9 and MDA-MB-231 cells compared to the MCF-7 cell line but only by about 2.0 fold.

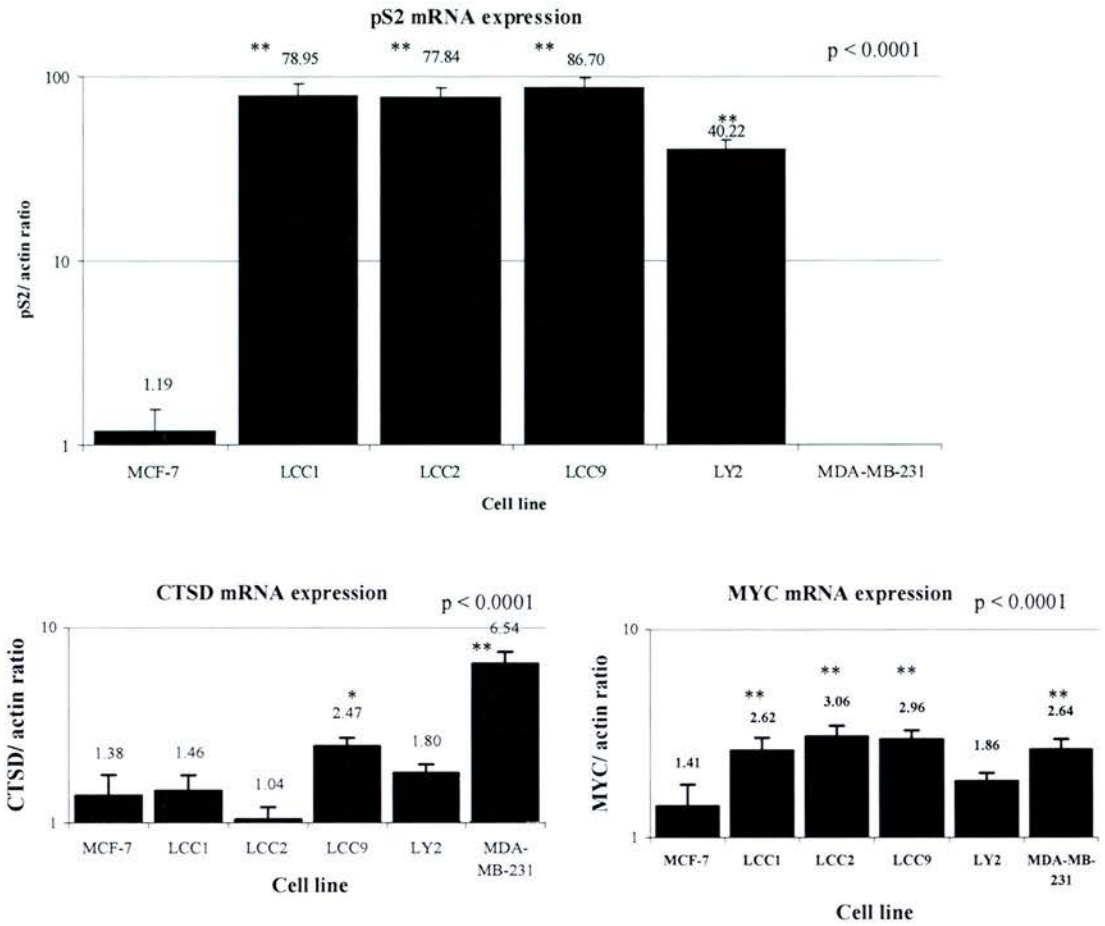


Figure 2.8: Baseline mRNA expression of pS2, CTSD and MYC before treatment (Oh) in breast cancer cells. MCF-7 cells seeded in complete media for 24h and a further 48h in reduced media containing 5% DCC. All other cells were seeded in reduced media for 24h before RNA collection. A representative experiment is shown of at least two experiments carried out. Each column presents the mean of triplicate PCR analysis for each sample. Error bars =STD. Significant variance between parental MCF-7 cells and other cell lines was determined by one-way ANOVA and Dunnets multiple comparison test where *=p<0.05, **=p<0.01.

2.3.3 Modulation of pS2, CTSD and MYC in MCF-7 cells

(i) E_2 stimulates pS2, CTSD and MYC mRNA expression in MCF-7 cells

Exposure of MCF-7 cells to E_2, resulted in stimulation of pS2, CTSD as well as MYC mRNA expression (Figure 2.9). pS2 mRNA expression increased after the first hour and continued to increase markedly up to 48h (120 fold). In contrast, the level of MYC mRNA remained elevated for 48h following an initial stimulation after 1h (3.7 fold). A continuous increase of mRNA was also detected for CTSD expression. The magnitude of increase relative to pS2 however was small. Maximum expression was reached at 48h (2.9 fold).

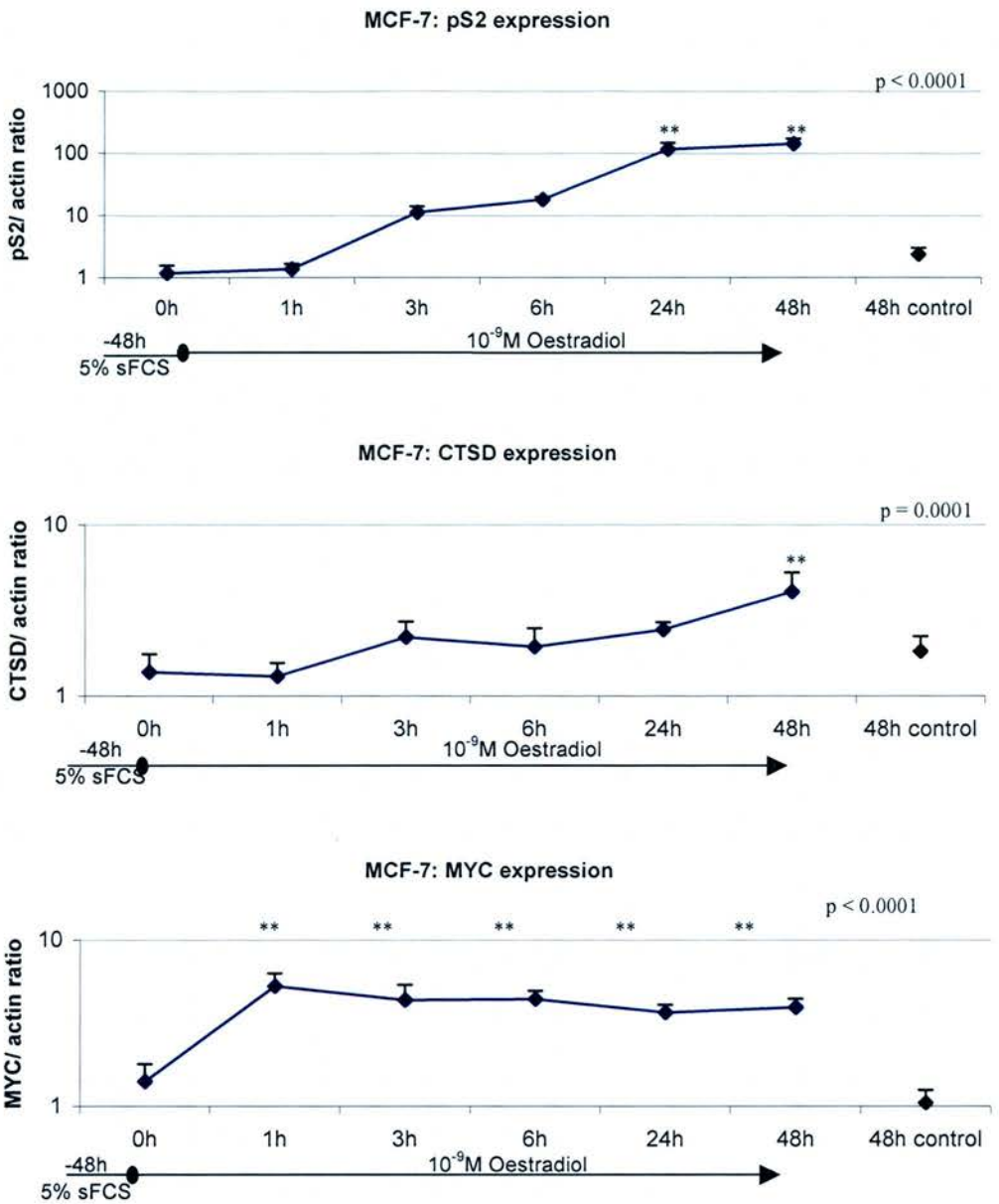


Figure 2.9: mRNA expression of pS2, CTSD and MYC in MCF-7 cells. Cells were left untreated (control group) or treated with 10^{-9}M E_2. RNA was collected at 0h, 1h, 3h, 6h, 24h and 48h. A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Error bars =STD. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=p<0.05, **=p<0.01.

(ii) Modulation of pS2, CTSD and MYC mRNA by E_2 and tamoxifen in MCF-7 cells at 6h and 48h

Oestrogen inducibility for all three genes was confirmed in this part of the experiment (figure 2.10). The antioestrogen tamoxifen showed generally little effect after 6h but acted as a weak agonist in some cases after 48h. pS2 mRNA expression was strongly induced by E_2 after 6h and 48h (90 fold and 666.8 fold, respectively). Tamoxifen as a single agent increased pS2 mRNA slightly and reached a 2.8 fold increase after 48h although statistical significance was not reached. Interestingly, tamoxifen suppressed the E_2 induced increase when combined with the E_2 at both time points and expression remained close to basal levels. A similar response to E_2 and tamoxifen was detected for the expression of MYC mRNA. E_2 treatment

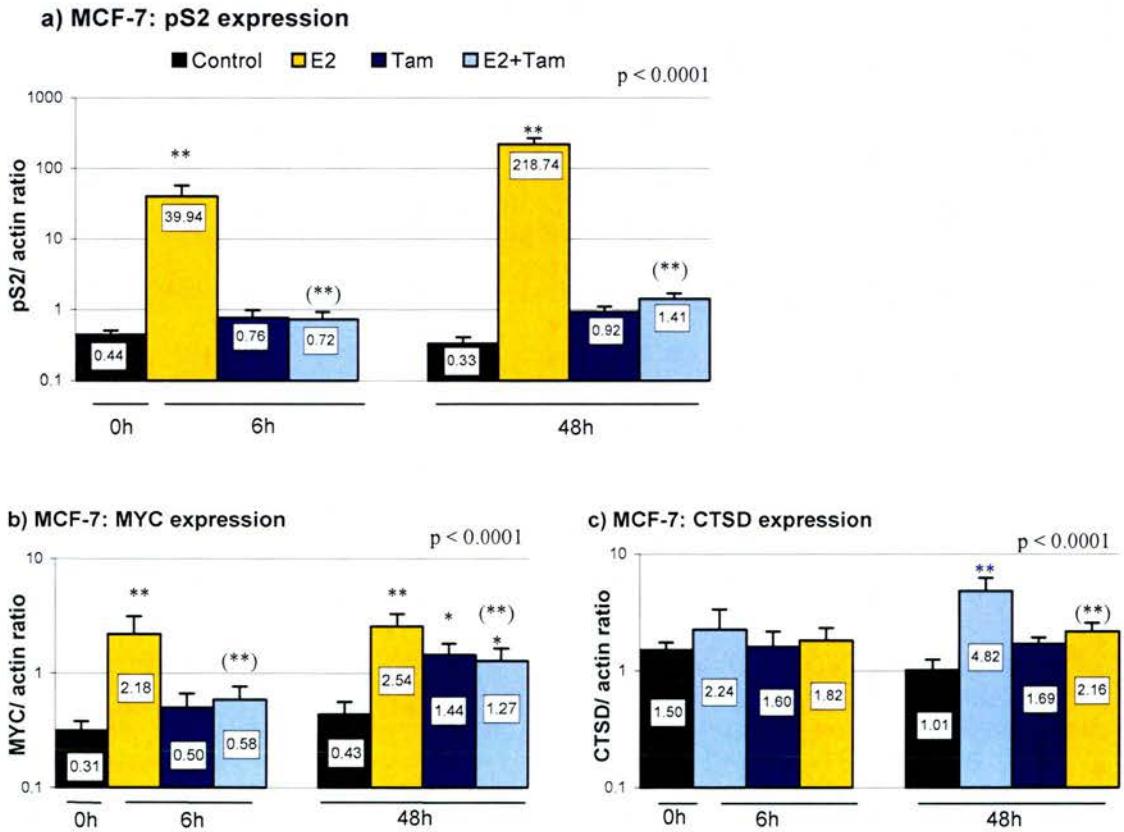


Figure 2.10: mRNA expression of pS2, CTSD and MYC in MCF-7 cells. Cells were left untreated (control group), treated with 10^{-9}M E_2; 10^{-6}M tam; or 10^{-9}M E_2 and 10^{-6}M tam. RNA was collected at 6h and 48h. A representative experiment is shown of at least two experiments carried out. Each column represents mean of triplicate PCR analysis for each sample demonstrating mRNA expression relative to actin expression. Error bars=STD. P-value for variance between all groups determined by one-way ANOVA and multiple Tukey-Kramer comparison test. Statistical significance noted for treatment groups compared to matched control where *=p<0.05, **=p<0.01. (**) indicates statistical significance comparing E+T with matched E_2.

markedly stimulated MYC expression at 6h (7.0 fold) as well as 48h (5.9 fold) while tamoxifen had slight agonistic effects reaching statistical significance at 48h (3.3 fold). Tamoxifen reduced the E_2 stimulated increase to basal levels at 6h and partially at 48h. As previously observed, E_2 increased CTSD expression at 6h but continued to increase expression with time reaching a 4.8 fold induction by 48h. Tamoxifen, by itself, had no effect on CTSD expression but reversed oestrogens stimulatory effect at 6h and 48h.

2.3.4 Oestrogen modulation of pS2, CTSD and MYC in LCC-1 cells

(i) Modulation of E_2 on pS2, CTSD and MYC mRNA expression over a period of 48h

In contrast to MCF-7 cells, a remarkably high constitutive pS2 expression in LCC-1 was detected from time point 0h (Figure 2.11 A). Both CTSD mRNA and MYC mRNA were detected at comparably low levels Figure 2.11 B). E_2 treatment produced a small increase in pS2 expression (between 1.5 fold and 1.6 fold) and very weak oestrogen stimulation over the 48h time span in CTSD and MYC expression. Noteworthy is a transient increase in MYC expression at 1h (3.5 fold).

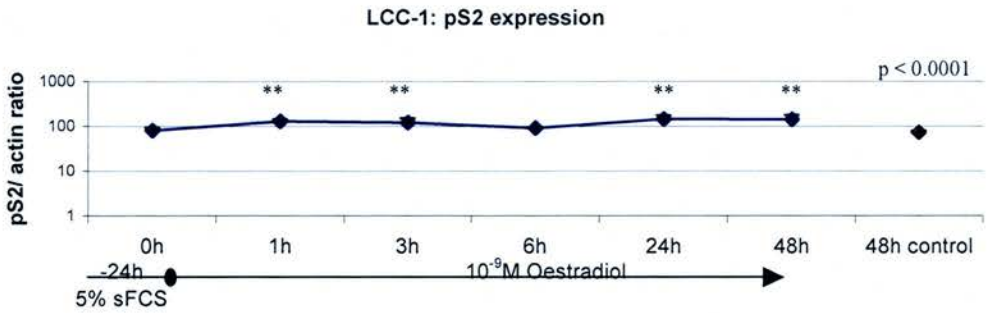


Figure 2.11A: mRNA expression of pS2 in LCC-1 cells. Cells were left untreated (control group) or treated with 10^{-9}M E_2. RNA was collected at 0h, 1h, 3h, 6h, 24h and 48h. A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Error bars =STD. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=$p<0.05$, **=$p<0.01$.

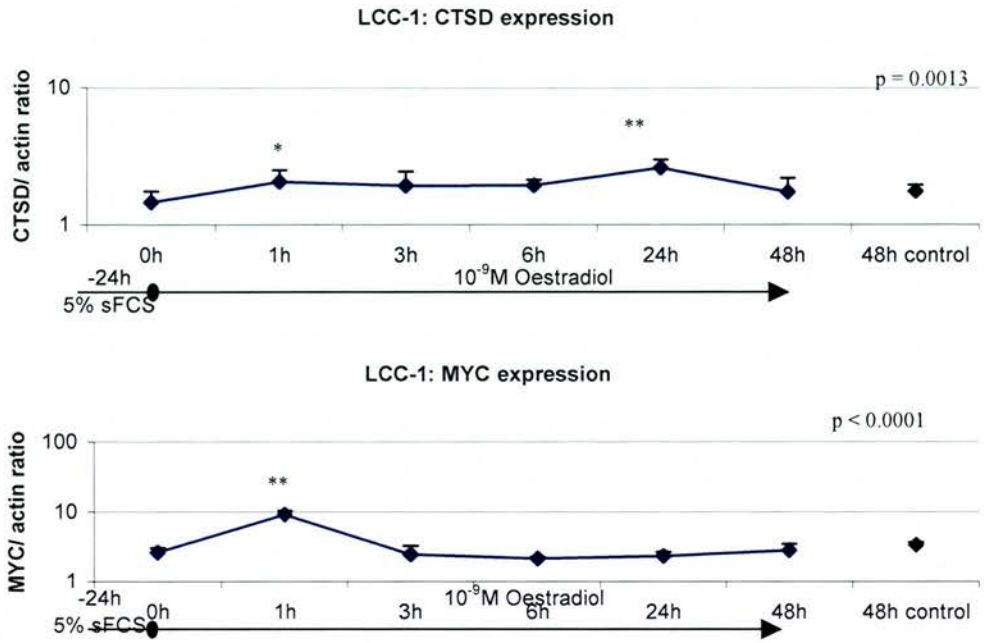


Figure 2.11: mRNA expression of CTSD and MYC in LCC-1 cells. Cells were left untreated (control group) or treated with 10^{-9}M E_2. RNA was collected at 0h, 1h, 3h, 6h, 24h and 48h. A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Error bars =STD. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=$p<0.05$, **=$p<0.01$.

(ii) Oestrogen and tamoxifen modulation on pS2, CTSD and MYC mRNA expression at 6h and 48h

As seen previously, pS2 was particularly strongly expressed in LCC-1 cells compared to MCF-7 cells and compared to the expression of CTSD and MYC (Figure 2.12). Interestingly, while E_2 led to an attenuated increase in pS2 mRNA expression (5.0 fold at 48h), tamoxifen alone as well as in combination with E_2 reduced expression short-term to below baseline levels (2.0 fold and 2.2 fold, respectively). This is not the case at 48h although tamoxifen does reduce the stimulatory effect of E_2. A similar observation was made for the transcription of MYC mRNA. E_2 stimulated levels at 6h (1.5 fold) and 48h (2.4 fold) while tamoxifen decreased mRNA levels strongly at 6h alone (4.8 fold) and in combination (3.6 fold). Levels remained below baseline in the presence of tamoxifen at 48h. Neither E_2 nor tamoxifen had a marked effect on CTSD expression.

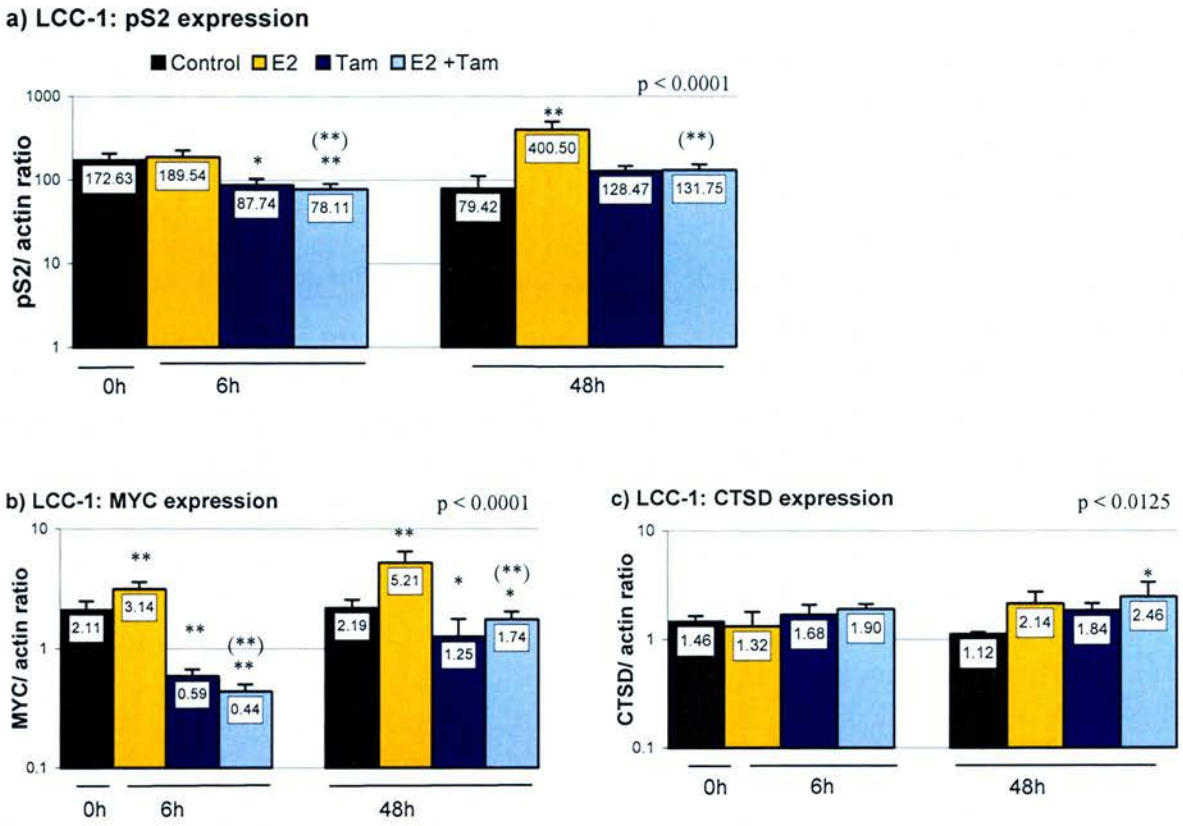


Figure 2.12: mRNA expression of pS2, CTSD and MYC in LCC-1cells. Cells were left untreated (control group), treated with 10^{-9}M E_2; 10^{-6}M tam; or 10^{-9}M E_2 and 10^{-6}M tam. RNA was collected at 6h and 48h. A representative experiment is shown of at least two experiments carried out. Each column represents mean of triplicate PCR analysis for each sample demonstrating mRNA expression relative to actin expression. Error bars=STD. P-value for variance between all groups determined by one-way ANOVA and multiple Tukey-Kramer comparison test. Statistical significance noted for treatment groups compared to matched control where *=p<0.05, **=p<0.01. (**) indicates statistical significance comparing E+T with matched E_2.

2.3.5 Oestrogen modulation of pS2, CTSD and MYC in LCC-2 cells

(i) Effect of E_2 on pS2, CTSD and MYC mRNA expression over a period of 48h

In a manner similar to LCC-1 cells, pS2, CTSD and MYC mRNA expression were shown to be only marginally oestrogen -inducible in LCC-2 cells when comparing to MCF-7 cells (figure 2.13). pS2 expression was again markedly high in LCC-2 cells (Figure 2.3.6). A small cumulative increase in expression was detected (2.4 fold at 48h). While CTSD mRNA was expressed at much lower levels than pS2, it was also weakly oestrogen inducible (2.8 fold at 24h). There was a significant short-term increase of MYC mRNA at 1h (2.2 fold) and a constitutive expression up

to 48h similar to basal levels. The immediate increase in expression after E_2 addition was also observed in LCC-1 cells.

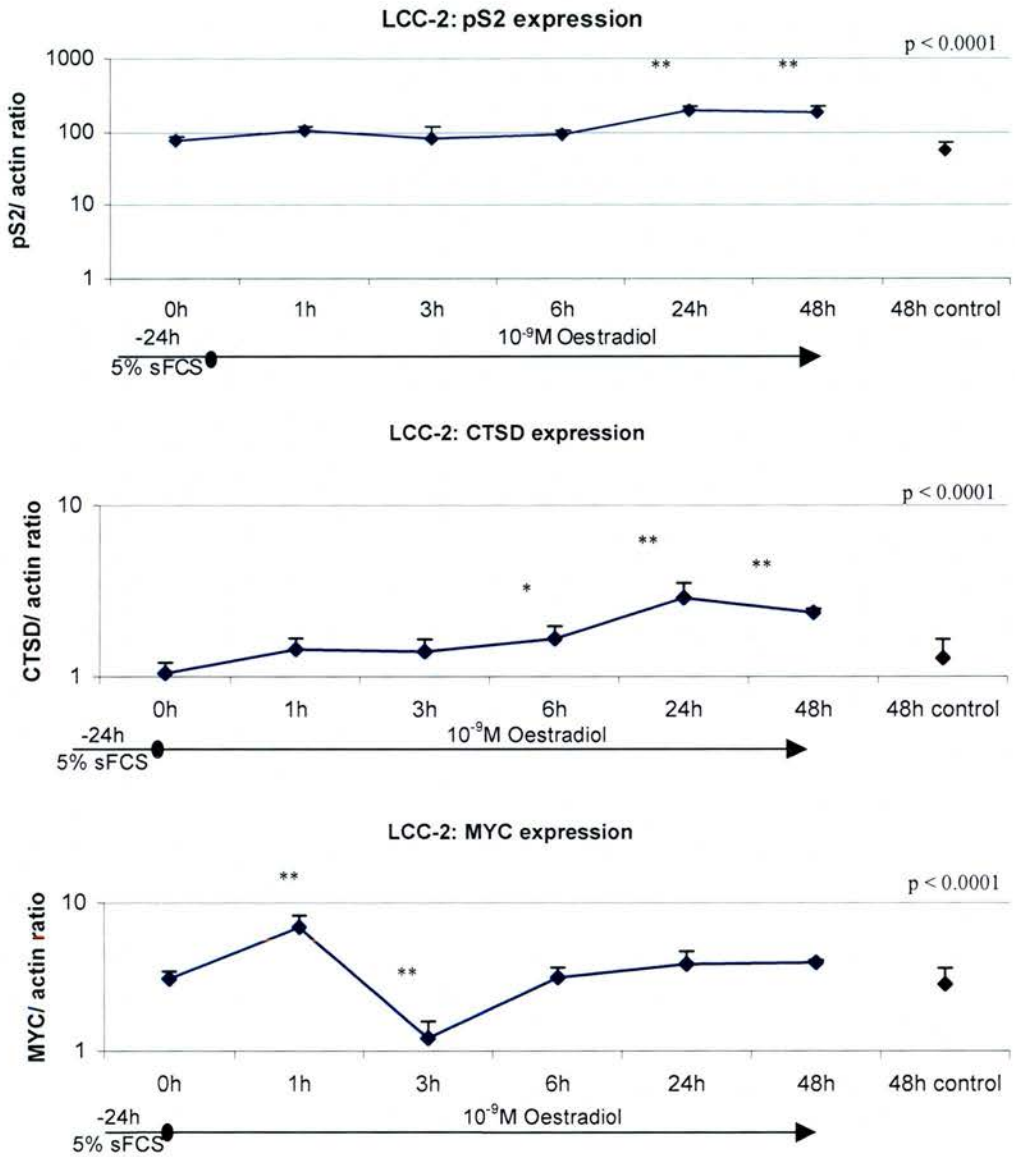


Figure 2.13: mRNA expression of pS2, CTSD and MYC in LCC-2 cells. Cells were left untreated (control group) or treated with 10^{-9}M E_2. RNA was collected at 0h, 1h, 3h, 6h, 24h and 48h. A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Error bars =STD. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=p<0.05, **=p<0.01.

(ii) Oestrogen and tamoxifen modulation of pS2, CTSD and MYC mRNA expression at 6h and 48h

When LCC-2 cells were subjected to oestrogen and tamoxifen, pS2 mRNA expression was found to be slightly stimulated at 6h and more strongly at 48h (1.5 fold and 2.5 fold, respectively) (Figure 2.14). Tamoxifen fully represses the stimulatory effects of E_2 at 48h. MYC and CTSD are expressed at very low levels compared to pS2. Treatment with E_2 only marginally increased the expression of MYC mRNA at 6h. Treatment with tamoxifen and tamoxifen in combination with E_2 reduces MYC mRNA levels below basal levels at 6h (4.0 fold and 3.4 fold, respectively). However, expression remains at control levels at 48h where neither E_2 nor tamoxifen have any effect. On the other hand, tamoxifen exerts an agonistic effect at 48h to increase CTSD mRNA expression (3.4 fold and 3.4 fold).

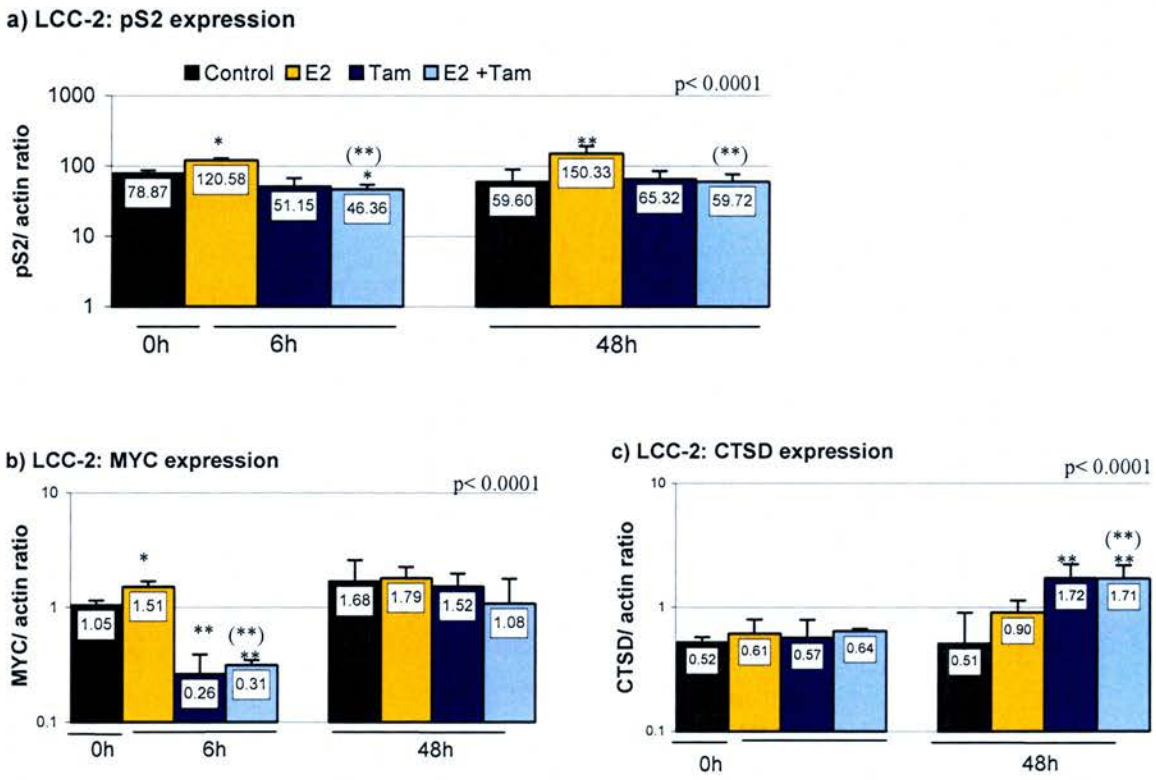


Figure 2.14: mRNA expression of pS2, CTSD and MYC in LCC-2cells. Cells were left untreated (control group), treated with 10^{-9}M E_2; 10^{-6}M tam; or 10^{-9}M E_2 and 10^{-6}M tam. RNA was collected at 6h and 48h. A representative experiment is shown of at least two experiments carried out. Each column represents mean of triplicate PCR analysis for each sample demonstrating mRNA expression relative to actin expression. Error bars=STD. P-value for variance between all groups determined by one-way ANOVA and multiple Tukey-Kramer comparison test. Statistical significance noted for treatment groups compared to matched control where *=p<0.05, **=p<0.01. (**) indicates statistical significance comparing E+T with matched E_2.

2.3.6 Modulation of pS2, CTSD and MYC in LCC-9 cells

(i) Oestrogen modulation of pS2, CTSD and MYC mRNA expression over a period of 48h

Even though pS2, CTSD and MYC mRNA were easily detectable in LCC-9 cells, E_2 treatment did not alter gene expression markedly compared to parental MCF-7 cells (Figure 2.15 A and B). Similar to LCC-1 and LCC-2 cells, pS2 expression was constitutively high and is slightly increased by 24h (1.5 fold) and 48h (1.7 fold). This expression pattern was also observed for MYC although the overall expression level was much lower compared to the expression of pS2. CTSD mRNA expression remains unaffected by oestrogen.

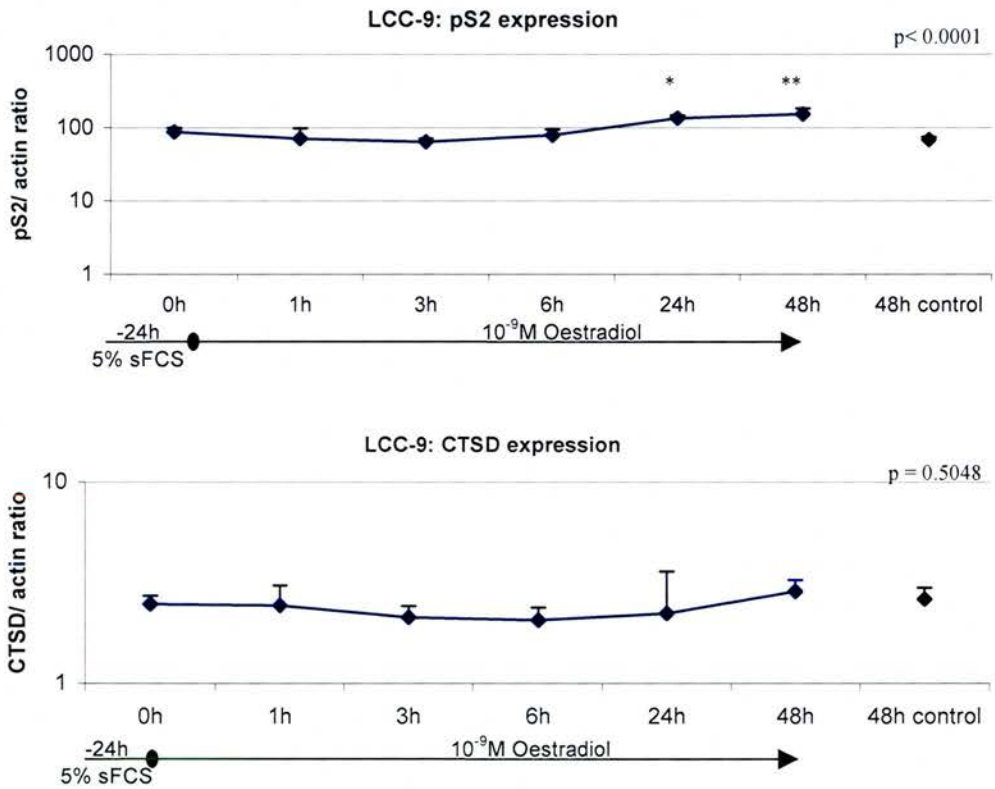


Figure 2.15 A: mRNA expression of pS2 and CTSD in LCC-9 cells. Cells were left untreated (control group) or treated with 10^{-9}M E_2. RNA was collected at 0h, 1h, 3h, 6h, 24h and 48h. A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Error bars =STD. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=$p<0.05$, **=$p<0.01$.

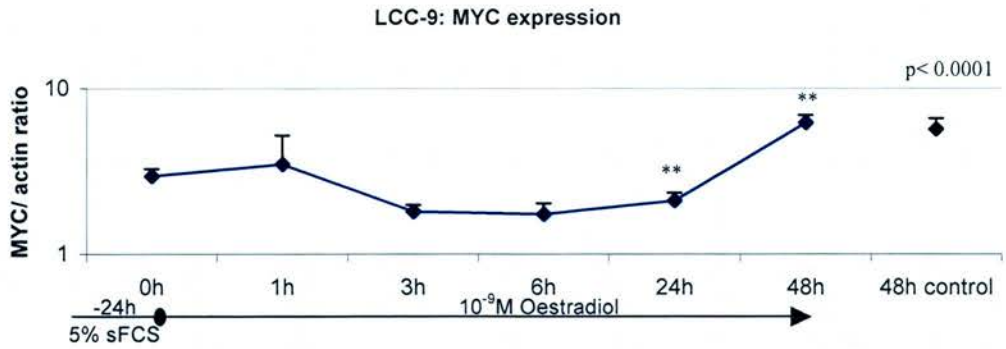


Figure 2.15 B: mRNA expression of MYC in LCC-9 cells. Cells were left untreated (control group) or treated with 10^{-9}M E_2. RNA was collected at 0h, 1h, 3h, 6h, 24h and 48h. A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Error bars =STD. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=p<0.05, **=p<0.01.

(ii) Oestrogen and tamoxifen modulation of pS2, CTSD and MYC mRNA expression at 6h and 48h

The expression pattern for pS2, MYC and CTSD mRNA was found to be similar in LCC-9 cells to LCC-2 cells (Figure 2.16 A and B). A late stimulatory effect on the expression of pS2 and CTSD was detected as seen previously at 48h (3.6 fold and 1.5 fold, respectively). Tamoxifen alone slightly reduced expression levels at 6h for pS2 and 6h as well as 48h for MYC (although not statistically

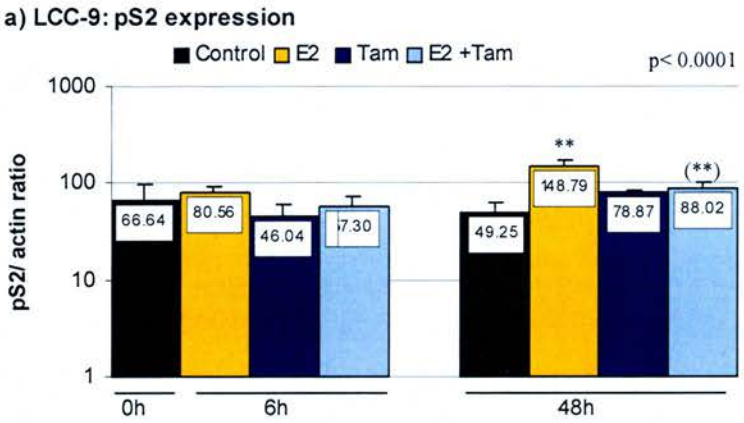


Figure 2.16 A: mRNA expression of pS2 in LCC-9cells. Cells were left untreated (control group), treated with 10^{-9}M E_2; 10^{-6}M tam; or 10^{-9}M E_2 and 10^{-6}M tam. RNA was collected at 6h and 48h. A representative experiment is shown of at least two experiments carried out. Each column presents mean of triplicate PCR analysis for each sample demonstrating mRNA expression relative to actin expression. Error bars=STD. P-value for variance between all groups determined by one-way ANOVA and multiple Tukey-Kramer comparison test. Statistical significance noted for treatment groups compared to matched control where *=p<0.05, **=p<0.01. (**) indicates statistical significance comparing E+T with matched E_2.

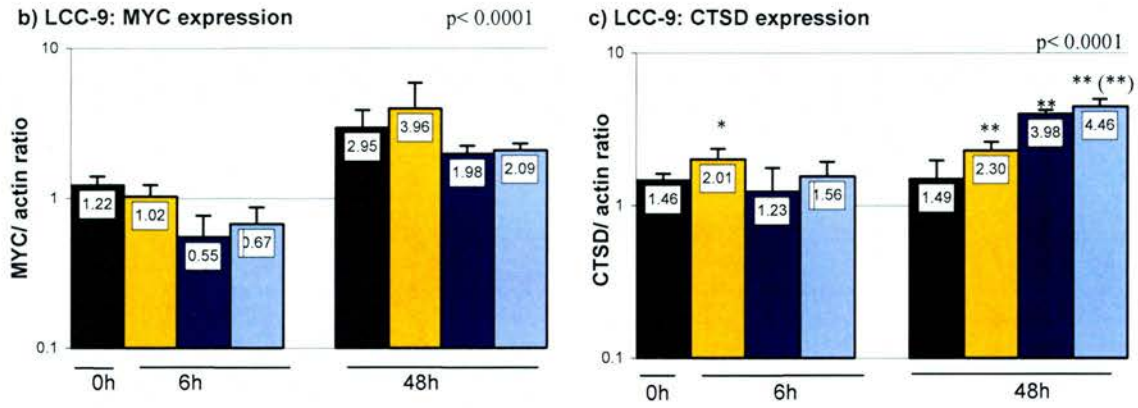


Figure 2.16 B: mRNA expression of CTSD and MYC in LCC-9cells. Cells were left untreated (control group), treated with 10^{-9}M E_2; 10^{-6}M tam; or 10^{-9}M E_2 and 10^{-6}M tam. RNA was collected at 6h and 48h. A representative experiment is shown of at least two experiments carried out. Each column presents mean of triplicate PCR analysis for each sample demonstrating mRNA expression relative to actin expression. Error bars=STD. P-value for variance between all groups determined by one-way ANOVA and multiple Tukey-Kramer comparison test. Statistical significance noted for treatment groups compared to matched control where *=p<0.05, **=p<0.01. (**) indicates statistical significance comparing E+T with matched E_2.

significant). Tamoxifen reduced the E_2 induced expression increase of pS2 significantly at 48h. The expression of CTSD was increased by E_2 at 6h as well as 48h (1.4 fold and 1.5 fold, respectively). Again, as seen in LCC-2 cells tamoxifen alone and in combination with E_2 revealed a late stimulation in LCC-9 cells. The expression was increased 2.7 fold and 3.0 fold, respectively.

2.3.7 Modulation of pS2, CTSD and MYC in LY-2 cells

(i) Modulation of pS2, CTSD and MYC mRNA expression over a period of 48h

The expression of pS2, CTSD and MYC was detected to be constitutively high in LY2 cells (Figure 2.17). A small increase in expression was observed for pS2 at 48h (2.4 fold). The decrease in expression for CTSD was observed at 6h and 48h (1.1 fold and 1.5 fold) and a small increase at 6h for MYC (1.3 fold). The expression of MYC mRNA returned back to baseline at 48h. More detailed analysis will have to be carried out to draw definite conclusions on the short and long-term effect of E_2 treatment.

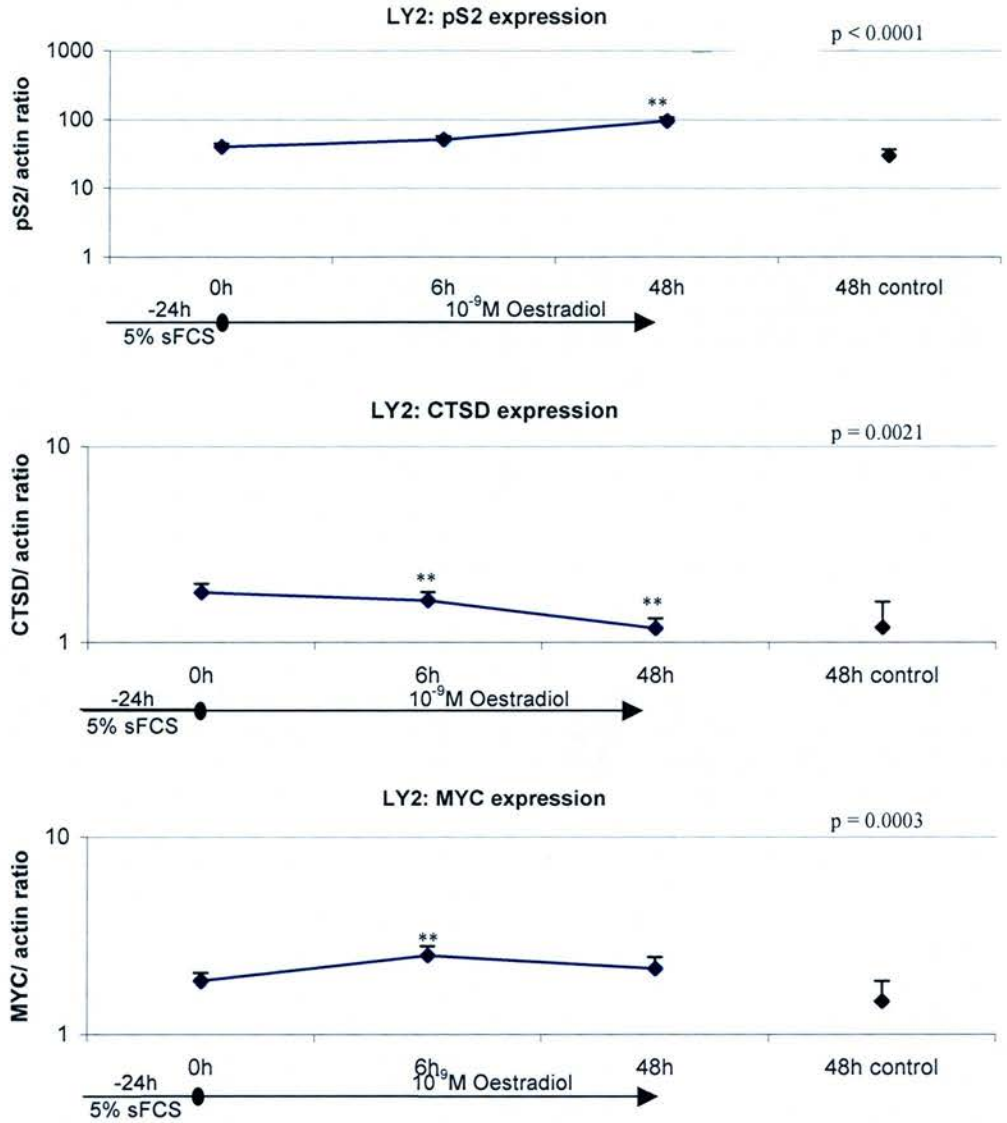


Figure 2.17: mRNA expression of pS2, CTSD and MYC in LY2 cells. Cells were left untreated (control group) or treated with 10^{-9}M E_2. RNA was collected at 0h, 6h and 48h. A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Error bars =STD. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=p<0.05, **=p<0.01.

(ii) Oestrogen and tamoxifen modulation of pS2, CTSD and MYC mRNA expression at 6h and 48h

Uniquely, tamoxifen treatment showed a significant decrease in expression for MYC and to a lesser extent for CTSD (figure 2.18). At 6h, MYC mRNA expression was reduced by tamoxifen alone and tamoxifen plus E_2 (5.5 fold and 4.1 fold). mRNA levels recovered by 48h. CTSD expression was reduced slightly at 6h (1.8 fold and 1.5 fold). However, this effect was reversed by 48h and tamoxifen increased expression in the presence of tamoxifen alone and in combination with E_2

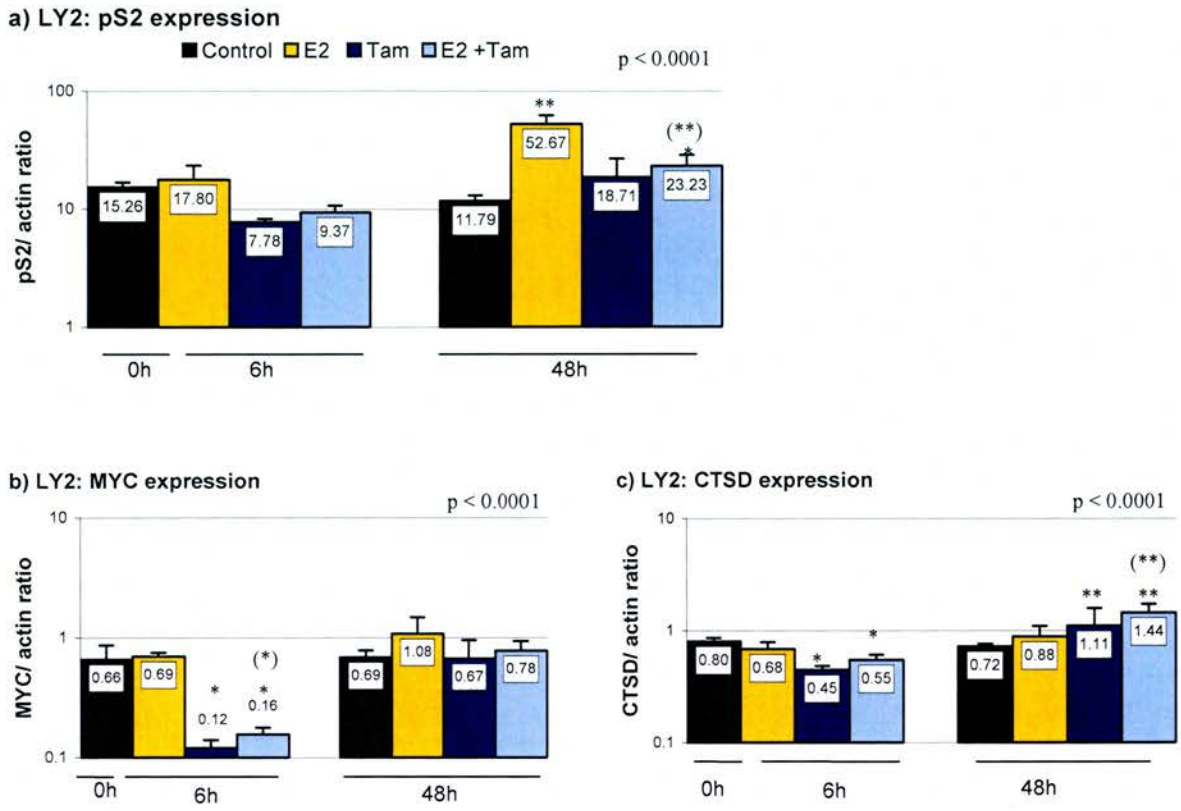


Figure 2.18: mRNA expression of pS2, CTSD and MYC in LY2 cells. Cells were left untreated (control group), treated with 10^{-9}M E_2; 10^{-6}M tam; or 10^{-9}M E_2 and 10^{-6}M tam. RNA was collected at 6h and 48h. A representative experiment is shown of at least two experiments carried out. Each column represents mean of triplicate PCR analysis for each sample demonstrating mRNA expression relative to actin expression. Error bars=STD. P-value for variance between all groups determined by one-way ANOVA and multiple Tukey-Kramer comparison test. Statistical significance noted for treatment groups compared to matched control where *=p<0.05, **=p<0.01. (**) indicates statistical significance comparing E+T with matched E_2.

(1.3 fold and 2.0 fold, respectively). E_2 stimulated pS2 mRNA levels at 48h as seen in the previous experiment (figure 2.17). This effect was partially reversed by the addition of tamoxifen.

2.3.8 Modulation of pS2, CTSD and MYC in MDA-MB-231 cells

(i) Oestrogen modulation of pS2, CTSD and MYC mRNA expression over a period of 48h

To compare the expression of pS2, CTSD and MYC to an ERα negative cell line, MDA-MB-231 cells were subjected to mRNA analysis (figure 2.19). As previously reported, pS2 mRNA could not be detected in these cells. E_2 exposure did not trigger transcription and had no effects on the expression of CTSD or MYC

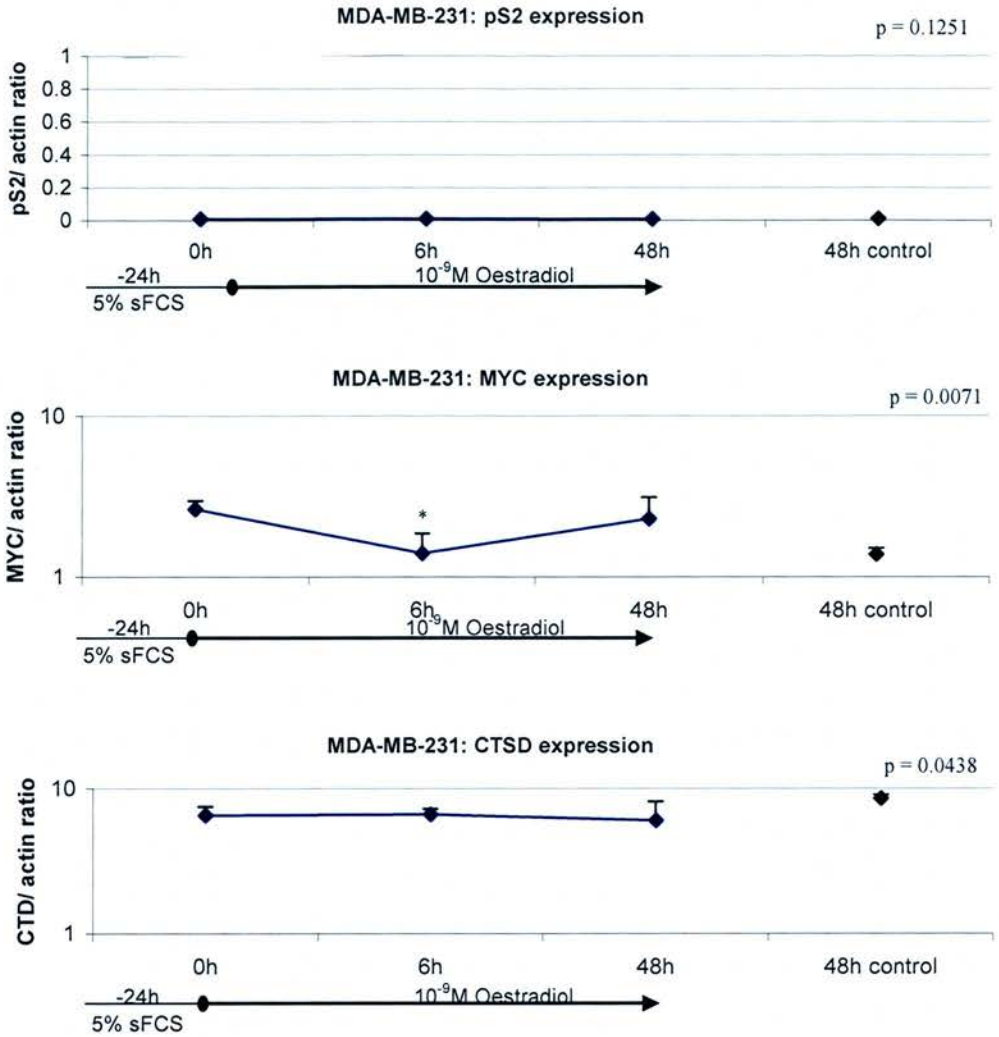


Figure 2.19: mRNA expression of pS2, CTSD and MYC in MDA-MB-231 cells. Cells were left untreated (control group) or treated with 10^{-9}M E_2. RNA was collected at 0h, 6h and 48h. Representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Error bars =STD. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=$p<0.05$, **=$p<0.01$.

although constitutively high levels were observed for both genes. The level of CTSD mRNA was relatively high compared to MCF-7 and MCF-7 variant cell lines.

Summary of E_2 and tamoxifen modulation on mRNA expression

	pS2				CTSD				MYC			
	C	E_2	Tam	E_2+ Tam	C	E_2	Tam	E_2+ Tam	C	E_2	Tam	E_2+ Tam
MCF-7	X	+++	+	+	X	+	+	+	X	+	-	-
LCC-1	XXX	+	-	-	X	-	-	+	XX	+	+	+
LCC-2	XXX	+	-	-	X	-	+	+	X	-	+	+
LCC-9	XXX	+	-	-	X	-	+	+	XX	-	+	-
LY2	XXX	+	-	-	X	-	+	+	X	-	+	+

Table 2.4: Summary of E_2 and tamoxifen modulation on mRNA expression in MCF-7 and MCF-7 variant cell lines at 48h. Baseline 48h mRNA expression analysis (C): Actin ratio is shown where x: <2; xx: 2-10; xxx: >10. Fold changes in response to E_2, tam and E_2+tam are indicated in comparison to matched basal levels. +++ = >50 fold; ++ = >10 fold; + = >2 fold; - = <2 fold; n.d. = not detected.

2.3.8 Discussion

Expression analysis at the transcriptional level has been carried out to examine the regulation of three oestrogen responsive genes: pS2, CTSD and MYC. The effects of oestrogen and tamoxifen have been studied to evaluate their role in endocrine response and also to identify a gene profile that best reflects observations in the cells' phenotypes. A summary of results is shown in table 2.4. Basal transcription of the three genes between cell lines varied to a great extent particularly for pS2. Here, mRNA expression was exceptionally high and comparable in LCC-1, LCC-2, LCC-9 and LY2 cells while MCF-7 cells showed a significantly lower expression. Differential expression of pS2 suggests a unique role for the gene in each of the cell lines. This role might be linked to altered endocrine phenotypes as all oestrogen resistant cell lines share high pS2 expression. MDA-MB-231 cells were pS2 negative implying that pS2 expression is linked to ERα signalling. This is not true for MYC mRNA expression in MDA-MB-231 cells where it was particularly strong compared to MCF-7 and LCC-1 cells as well as LCC-9 and LY2 cells. In MCF-7 and MCF-7 variant cell lines, only small baseline expression differences were detected for CTSD and MYC. Cathepsin D was generally expressed at lower levels comparable to MYC. Most dramatic was the stimulatory effect of oestrogen seen in MCF-7 cells although some stimulation for this gene was seen in all other cell lines. Analysis of baseline expression and E_2 modulation indicates a clear

oestrogen driven pS2 expression in MCF-7 cells but also suggests high pS2 expression in the MCF-7 variant lines is largely driven in an E_2 independent manner. It might also imply that any remaining stimulation of pS2 expression bypasses ERα and oestrogen regulates the expression through an indirect signalling pathway. Oestrogen induced transcription of MYC and CTSD mRNA significantly on relatively few occasions, most notably the expression of MYC in MCF-7 and LCC-1 cells. Interestingly, tamoxifen has a late stimulatory effect for CTSD expression particularly in LCC-2, LCC-9 and LY2 cells. The antioestrogen generally also reversed the stimulation of oestrogen in all but MDA-MB-231 cells whenever an induction was observed such as in pS2 expression, or MYC expression in LCC-1 cells. Additionally, E_2 induced MYC expression was not only reversed by tamoxifen but reduced below baseline levels. This is clearly an indication that oestrogen and tamoxifen have cell line and promoter specific effects on gene transcription. Both, oesrteogen as well as tamoxifen can show agonistic and antagonistic effects on mRNA expression and tamoxifen is clearly not always able to reverse the effects of oestrogen.

Studies investigating the clinical relevance of pS2/TFF1 have established a positive correlation between pS2 and oestrogen receptor expression. This clearly suggested that the expression of pS2 in breast carcinomas is oestrogen dependent (reviewed in Ribieras, S. *et al.* 1998). Multiple *in vitro* studies have produced evidence that pS2 gene transcription is oestrogen inducible at the mRNA as well as protein level in MCF-7 cells producing accumulating high levels of pS2 mRNA and protein in the presence of the hormone (Masiakowski, P. *et al.* 1982, Jakowlew, S.B. *et al.* 1984 and Nunez, A.M. *et al.* 1987). Structural analysis has shown that the clover-like organization of the pS2 protein is compact and stable as it is resistant to proteases and thiol agents (reviewed in May, F.E.B. *et al.* 1997). Little or no pS2 expression has been observed in the hormone free environment. A more recent study also confirms the immediate and continuous inducibility of pS2 mRNA as a significant increase in expression is reported after just 1h and up to 24h (Kim, J. *et al.* 2000). In more detail, *in vivo* DNase I footprinting studies gave insight into the involvement of the ERE of pS2 located within the promoter. Both ERE half sites, the consensus as well as the imperfect sites, and adjacent nucleotide sequences appeared

to be protein protected indicating extensive protein binding and gene interaction. Importantly, the oestrogen receptor proved to be part of this protein complex. In contrast, only the consensus site was shown to be protected in the absence of the hormone. Tamoxifen has been shown to be weakly agonistic influencing the expression of pS2 mRNA but effective in reversing oestrogen induced expression levels (Weaver, C.A. *et al.* 1988, Osborne, C.K. *et al.* 1995). This is supported by the observation that minimal ERE protein binding takes place in MCF-7 cells treated with tamoxifen (Kim, J. *et al.* 2000). On the other hand, the pure antioestrogen ICI182,780 (not studied in this experiment) demonstrated intensive and distinct ERE protection. Taken together, these observations suggest that the pS2 gene is not only clearly, oestrogen regulated but distinct use of ERE half sites in the form of protein recruitment enables differential response to oestrogen and antioestrogens, and pS2 transcription.

pS2/TFF1 is expressed in ER positive but not ER negative breast cancer cell lines (May, F.E.B. and Westley, B.R. 1986 and 1988). This was confirmed in the experiment comparing ER positive MCF-7 variant cell lines with ER negative MDA-MB-231 cells. Additionally, expression differences of pS2 protein have been demonstrated between ER positive MCF-7, T47D and ZR75 cells (Prest, S.J. *et al.* 2002). Here, expression of the gene was particularly strong in LCC-1/2/9 and LY2 cells compared to MCF-7 cells. These results might indicate a link between strong pS2 expression and a more advanced breast cancer phenotype as seen in the MCF-7 variant lines. Trefoil factor proteins are thought to be involved in homeostasis and repair of damaged tissue through motogene functions (reviewed in Ribieras, S. *et al.* 1998). Observations were first made in the gastrointestinal tract. TFFs stimulate the mobilization of epithelial cells surrounding damaged area to resurface the epithelium. This concept was also applied for investigations in breast cancer cells. Evidence suggests that TFF1/pS2 concentrations detected in the cell medium but also TFF2 stimulate MCF-7 cell movement perhaps through interaction of pS2 with to date unknown cell surface receptor (Prest, S.J. *et al.* 2002 and May, F.E.B. *et al.* 2004). The two molecular forms of the protein are expressed equally but the homodimer appeared to stimulate at lower pS2 concentrations than the monomer. The presence of two different pS2 forms and the concentration providing sensitive cell motility

may hint at an explanation for the role of different expression levels of pS2 in breast cancer cell lines. If pS2 encompasses motogenic functions, then cells expressing this factor are able to spread more easily and potentially exhibit increased invasiveness compared to normal tissue. In oestrogen sensitive breast cancer cells, exposure to the hormone might consequently lead to enhanced cancer progression through pS2 mediated cell movement.

However, oestrogen exposure is not the mediator in oestrogen insensitive breast cancer such as LCC-9 and LY2 cells. Transcription of pS2 could partially be mediated by cross-talk to growth factor or protein kinase pathways as suggested for the signalling networks of ERα or CTSD (Gruber, C.J. *et al.* 2002; Laurent-Matha, V. *et al.* 2005). Also, recombinant pS2 stimulated cell movement was demonstrated in MDA-MB-231 cells despite their lack of pS2 expression (Prest, S.J. et al. 2002). Migration was stimulated at higher pS2 concentration than the pS2 expressing tissue suggesting a lower sensitivity to pS2 in this cell line. This implies a process by which a breast cancer cell deficient of pS2 can reach a cancerous state characterized by enhanced metastatic properties.

The absence of detectable levels of pS2 mRNA in MDA-MB-231 could also point to a loss of pS2 function in growth factor signalling in some malignant breast tissue. pS2 inactivated mouse models have revealed that lack of the protein leads to abnormal cell proliferation and differentiation in gastric tissue (Lefebvre, O. *et al.* 1996). The gene has therefore been suggested to acts as a tumor suppressor in this form of cancer. Supportive of this proposition, it has been determined multiple times that progression of normal gastric epithelial cells to a malignant phenotype can include the loss of pS2 expression (reviewed in Ribieras, S. *et al.* 1998). In addition, normal surrounding epithelium can remain pS2 positive while expression is absent in malignant tissue. Molecular mechanisms for such observations remain elusive and similar detailed investigations in breast cancer are rare. In the breast it is known that pS2 is expressed in many mammary carcinomas but not in normal tissue (Rio, M.C. *et al.* 1987 and Jakowlew, S.B *et al.* 1984).

Overexpression of cathepsin D in breast cancer has long been associated with an increased risk of clinical metastasis and shorter survival (reviewed in Rocherfort, H. *et al.* 2000). Many investigations have since focused on the molecular

mechanisms leading to the overexpression and its role in cancer development. RT-PCR analysis has demonstrated that MCF-7 cells and all MCF-7 variant lines express CTSD mRNA at similar levels. Interestingly, the ER negative MDA-MB-231 cell line not only expressed CTSD but also showed levels higher than the other cell lines. Oestrogen had slight long-term stimulatory effects in MCF-7 cells and LCC-9 cells. The antioestrogen tamoxifen had differing effects on CTSD transcription. In MCF-7 cells, oestrogen induced expression was reversed. In contrast, tamoxifen acted as agonist and revealed an approximately 2-3 fold increase in mRNA expression in LCC-2, LCC-9 and LY2 cells.

There is extensive evidence that CTSD mRNA and protein are expressed not only in ER positive but also ER negative breast cancer cell lines (Cavailles, V. *et al.* 1988 and 1989, Westley, B. and Rochefort, H. 1980 and others). Furthermore, oestrogen has been shown to induce mRNA and protein expression in ER positive breast cancer cells at E_2 concentration levels sufficient to fully occupy the receptor, implying an ER mediated event in these cells (Westley, B.R. and May, F.E.B. 1987). The oestrogen regulation has been shown to be due to binding of the ER dimer to an oestrogen response element identified in the promoter of the CTSD gene (Augerau, P. *et al.* 1994). Oestrogen induced CTSD mRNA transcription after longterm exposure in MCF-7 cells in this experiment. However, in the ER positive but oestrogen unresponsive LCC-2, LCC-9 and LY2 cells, the same principle as in ER negative cells such as the MDA-MB-231 may apply. Previous studies have shown that CTSD is expressed in ER negative breast tumour cell lines BT 20 and MDA-MB-231 but not regulated by oestrogen (Westley, B.R. and May, F.E.B. 1987). That gives reason to believe that the ER is either activated in a ligand independent manner or bypassed and cross-talk with other pathways takes place. Other factors such as EGF and insulin have been shown to play a role in CTSD activation (Cavailles, V. *et al.* 1988). Alternative pathways circumventing the ER will be in place in oestrogen unresponsive as well as oestrogen receptor negative cells. Antioestrogen exposure has previously shown to have no effect on CTSD expression in MCF-7 cells, although, it did reverse oestrogen stimulated expression (Westley, B. and Rochefort, H. 1980). As seen with other oestrogen-regulated genes, tamoxifen exerts its partial agonistic properties when inducing CTSD expression in oestrogen responsive tissues.

This had been reported in LY2 cells (Cavailles, V. *et al.* 1988). Reasons for the induction are unknown.

Despite the consistent expression of CTSD across the breast cancer cell panel, the relatively small effects of oestrogen and tamoxifen might at first glance make this a less interesting gene for breast cancer research. However, recent studies present strong arguments for its role in breast cancer tumor progression. Mitogenic characteristics of CTSD were first established in MCF-7 cells (Vignon, F. *et al.* 1986). Two forms of CTSD, the catalytically active mature protein cath-D and an inactive precursor pro-cath-D have been reported to be expressed *in vitro* and *in vivo* and to be involved in cell proliferation and angiogenesis (Berchem, G. *et al.* 2002 and references within). In addition, CTSD overexpressed in epithelial cancer cells might signal to neighbouring stromal fibroblasts stimulating cell growth, survival, motility and invasion (Laurent-Matha, V. *et al.* 2005). This fibroblast activation could be an essential step for tissue surrounding the primary tumour site to become more invasive and proliferative. Mechanistically, the involvement of a cell surface receptor responding to the release of CTSD seems plausible. The only known receptors to interact with CTSD are two types of Man-6-P/IGF2 receptors (Rochefort, H. *et al.* 2000). However, conflicting evidence of their mediation has lead to suggestions of the involvement of an unidentified receptor and cross-activation through the complex MAPK pathway (Glondu, M. *et al.* 2001; Berchem, G. *et al.* 2002; Laurent-Matha, V. *et al.* 2005). Taken together, this evidence suggests a role for CTSD in the development of breast cancer. More research is essential to uncover its potential as a therapeutic target.

MYC plays a vital role in cell proliferation and growth by affecting many aspects of cell cycle regulation, apoptosis, metabolism, differentiation and cell adhesion (Dang, C.V. *et al.* 1999). The involvement in breast cancer has been demonstrated extensively (Escot, C. *et al.* 1986; Berns, E.M. *et al.* 1992, 1996; and others). In malignant tissue, over-expression of MYC is thought to be due to a loss of down-regulation leading to constitutive gene expression (reviewed in Grandori, C. *et al.* 2000 and Pelengaris, S. *et al.* 2002). Growth factor independence has been reported as a result of MYC over-expression. Oestrogen has been shown to stimulate MYC mRNA and protein expression in ER positive breast cancer cells including

MCF-7 cells (Dubik, D. *et al.* 1987 and Watson, P.H. *e al.* 1991). Malignant and non-malignant ER negative cells have been shown to express MYC to varying levels but remain unaffected by oestrogen and some antioestrogens including tamoxifen. In this experiment MYC mRNA expression in ER positive and ER negative breast cancer cell lines was also observed. Lower baseline levels were detected in ER positive MCF-7 and LY2 cells compared to ER positive LCC-1/2/9 and ER negative MDA-MB-231 cells. Strong oestrogen inducibility was confirmed in MCF-7 cells and to a lesser degree in LCC-1 cells. Interestingly, tamoxifen reduced mRNA expression in LCC-1 cells on its own and reduced the oestrogen stimulation when combined with the hormone. The antieostrogen had a slight delayed stimulatory effect in MCF-7 cells. Generally, constitutively high expression of MYC mRNA was observed in LCC-2 and LCC-9 cells unaffected by oestrogen or tamoxifen exposure.

Antisense oligonucleotide experiments have been shown to inhibit E_2 stimulated cell growth in MCF-7 cells giving MYC a central role in breast cancer cell growth (Watson, P.H. *et al.* 1991). Further experiments utilising RNAi directed against MYC indicate a decreased cell growth rate and colony formation as well as an increased sensitivity to apoptosis *in vitro* (Wang, Y. *et al.* 2005). MYC RNAi led to tumour inhibition longterm *in vivo* and *in vitro* after short RNAi exposure despite complete recovery of MYC protein levels. This indicated that a sustained loss of malignant characteristics could be caused by brief c-myc inhibition. However, there is also evidence that suggests that a continuous overexpression of MYC is necessary for tumour maintenance (Pelengaris, S. *et al.* 1999 and others). Whether the effects of MYC are reversible or not, the immediate change in malignant phenotype does indicate alternate pathways come into place immediately for MYC to exert its effects. One such pathway coming into effect in response to deregulated expression of MYC might involve the activation of the *ras* pathway. *RAS* had been suggested to inhibit MYC protein degradation resulting in accumulation of MYC activity (Sears, R. *et al.* 1999). About half the tumours of another study showed *KRAS2* mutations, a member of the *ras* proto-oncogene family (D'Cruz, C. *et al.* 2001). This group was unable to show tumour regression after reinduction of MYC expression whereas tumours lacking the *KRAS2* mutation did regress after MYC reinduction. Also, a fraction of mutation lacking group did recur after weeks or month hinting at a

selection process of cells through the heterogeneity of the tissue. Pathways such as the Ras/Raf/MAP Kinase or P13K pathway have been linked to MYC function on several occasions (for example: Mawson, A. *et al.* 2005). Taken together, the tumors are able to switch to preferred secondary oncogenic pathways, MYC independent growth mechanisms, leading to more malignant hormone and other external signal independent phenotypes as seen in LCC-2 and LCC-9 cells. The large range of mitogens such as IGF1, TGF-β and E_2 have shown to influence MYC expression and the increasing number of factors associating with MYC such as AP-2 or BRCA1 supports this theory (Dang, C. *et al.* 1999; Pelengaris, S. *et al.* 2002).

The malfunction of MYC is thought to be a result of failure of down-regulation of the myc gene as opposed to altered function the MYC protein. Relatively rare genomic alterations such as retroviral transductions, amplifications or chromosomal translocations have been identified (reviewed in Grandori, C. *et al.* 2000). Such alterations are thought to cause myc over-expression by increasing MYC mRNA levels by enhanced transcription initiation, decreased transcription attentuation and mRNA stability. In normal tissue, MYC expression levels are low and protein has a short half-live. Studies have suggested that MYC mutations might lead to altered protein stability (Salghetti, S.E. *et al.* 1999). It is a suggested that an ubiquitin-mediated proteolysis mechanism comes into place and MYC protein accumulates stimulating malignant cell proliferation. Despite the identification of such mutations, it remains questionable if they directly lead to altered cell proliferation, differentiation or apoptosis. Another possibility is that high levels of MYC expression are required for malignant development in cooperation with secondary mutations in MYC target genes. MYC amplification may induce genomic instability in such genes. This has been reported for the cyclin D2 gene (Mai, S. *et al.* 1999). Human and mouse B-lymphocytic tumour cells exhibiting amplified levels of MYC demonstrated an instability of the cell cycle regulating protein suggesting that elevated levels of cyclin D2 might be involved in tumour development. Genomic instabilities caused by MYC over-expression have been identified for other genes, for example the ribonucleotide reductase R2 gene (Kuschak, T.I. *et al.* 1999). The cooperation with other oncogenes as a result of constitutive MYC expression might enable the cell to fully avoid normal myc regulated cell proliferation. Again, a

rapidly dividing more malignant phenotype is the result. The complexity of MYC regulated cell growth makes it unlikely that targeting the MYC pathway only will have any potential benefit for breast cancer treatment.

There is no doubt that all three genes investigated in this part of the study are involved in malignant development of breast cancer and are therefore of potential value as therapeutic targets for breast cancer treatment. They have demonstrated unique expression patterns in response to oestrogen and tamoxifen. Results also suggest that these genes are expressed and regulated in a cell line and promoter specific manner. It also suggests that regulation does not simply take place through direct ER-ERE interaction within the target promoter but alternative elements such as the E-box as the interaction site within MYC or the receptors alternative activation site AF- 1. In addition, several other transcriptional cofactors might serve as links between the receptor and the promoter. The cooperativity between the ER signaling pathway to other growth factors pathways takes a vital role in differential gene regulation as discussed in subsequent chapters.

All three candidate genes show at least some reflection of the phenotype with respect to cell growth in the different cell lines. Because the changes in mRNA expression observed in response to oestrogen and tamoxifen were most consistent and dramatic for pS2, the gene was chosen for more detailed studies in this project.

2.4 Regulation of coactivators and corepressors by oestrogen and tamoxifen

2.4.1 Introduction

Oestrogen receptor activity has been associated with cofactors that can mediate either transcriptional activation or suppression. To determine a potential role for cofactors in ERα transcription, mRNA expression of coactivators of the p160 family, p300 and RIP140 as well as corepressors NCoR and SMRT were analysed in MCF-7 cells treated with 10^{-9}M E_2 at multiple time points up to 48h. Furthermore, the cells were subjected to 10^{-9}M E_2, 10^{-6}M tamoxifen and the combination of E_2 and tamoxifen for two periods of exposure, 6h and 48h and mRNA expression was investigated. Protein expression of the SRC-1/2/3, RIP140 and REA was investigated at 48h. Results were compared to the analysis of LCC-1, LCC-2, LCC-9, LY2 and MDA-MB-231 cells to establish possible characteristic expression patterns and detect whether a balance of these cofactors could reflect the cell line specific endocrine responses. Analysis of NCoR and SMRT protein could not be carried out. Testing of several commercially available antibodies did not identify one that detected bands representing the proteins clearly distinguishable from other proteins on a Western blot. NCoR with a size of about 270kDa and SMRT with a size of about 270, 180 and 80kDa are large proteins which makes separation on ordinary Western blots difficult.

Figure 2.20 compares cofactor expression in MCF-7, MCF-7 variant and MDA-MB-231 cells in the absence of oestrogen and tamoxifen. Coactivators and corepressors are expressed at comparable levels in MCF-7 cells. Differential expression of cofactor mRNAs have been detected for variant lines. Within the p160 family, SRC-3 showed reduced levels of expression in all variant cell lines and in MDA-MB-231 compared to the parental line. In MDA-MB-231 cells, SRC-1 and SRC-2 expression was also relatively low. LCC-1, LCC-2 and LCC-9 cells showed a paticularly high RIP140. These results could suggest a role for cofactors such as SRC-3 or RIP140 in the development of hormone resistant phenotypes.

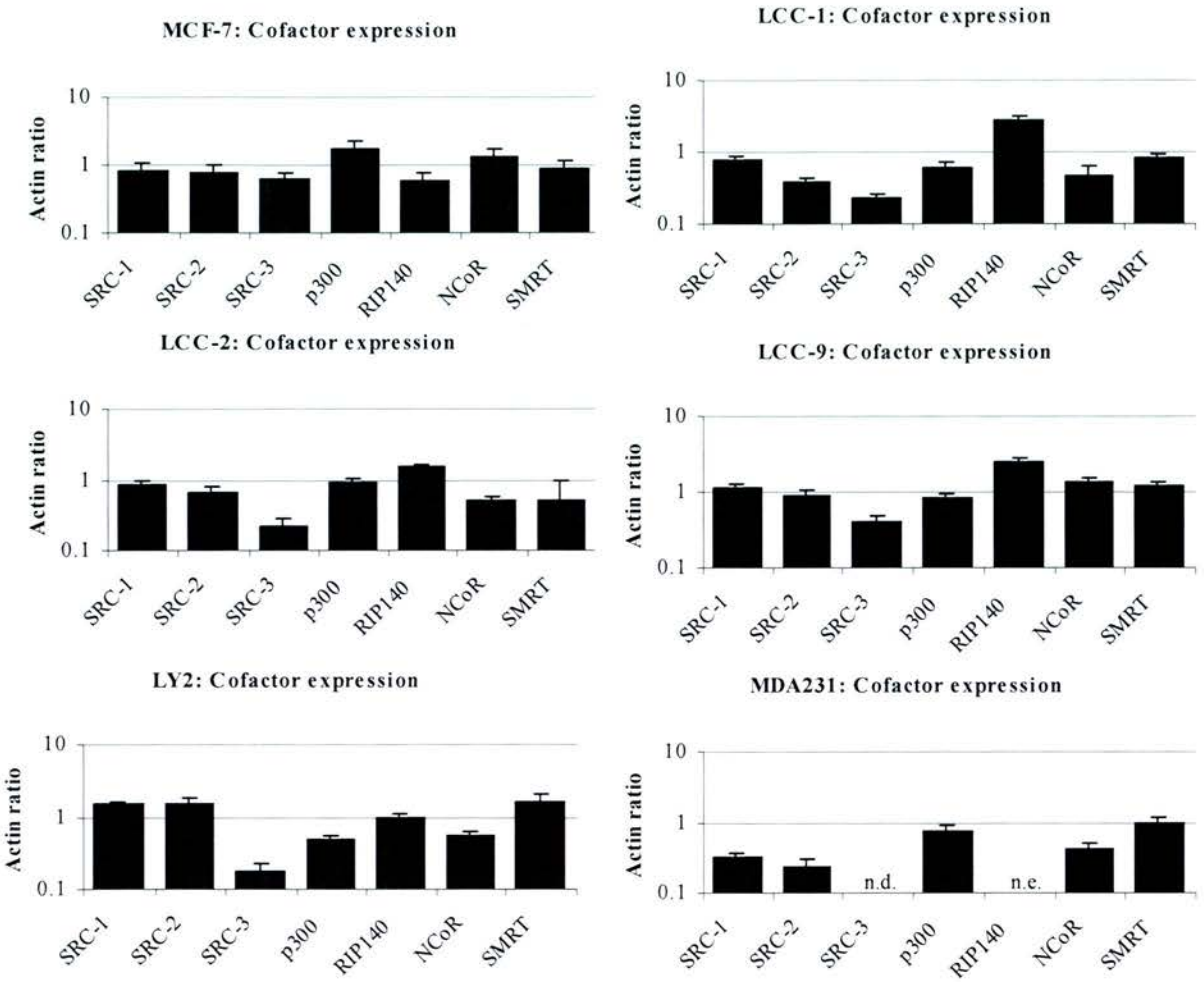


Figure 2.20: Cofactor mRNA expression in MCF-7 and MCF-7 variant cells. Cells were seeded in complete media for 24h and a further 48h in reduced media; all other cells were plated in reduced media for 24h before RNA collection. Each column represents the mean of triplicate PCR analysis. Error bars =STD; n.d.=not detectable; n.e.= not examined.

2.4.2 Modulation of coactivators and corepressors in MCF-7 cells

(i) Effect of E_2 on cofactor mRNA expression in MCF-7 cells

MCF-7 cells showed very similar levels of all analysed cofactors (Figure 2.21 A-C). Overall, the addition of oestrogen did not markedly regulate mRNA expression although minor changes were detected. Most notable was a common if subtle down-regulation at 24h and subsequent recovery by 48h. Highest expression was found for p300 mRNA. Expression was constitutive but an expression reduction (1.5 fold) was observed at 24h compared to 0h. This reduction was found to be similar for members of the p160 family but strongest in SRC-1 (2.5 fold). An oestrogen stimulated increase in any p160 factor mRNA was not observed. One of the corepressors, SMRT, demonstrated a small E_2 up-regulation by 3h (1.2 fold), although it did not reach statistical significance.

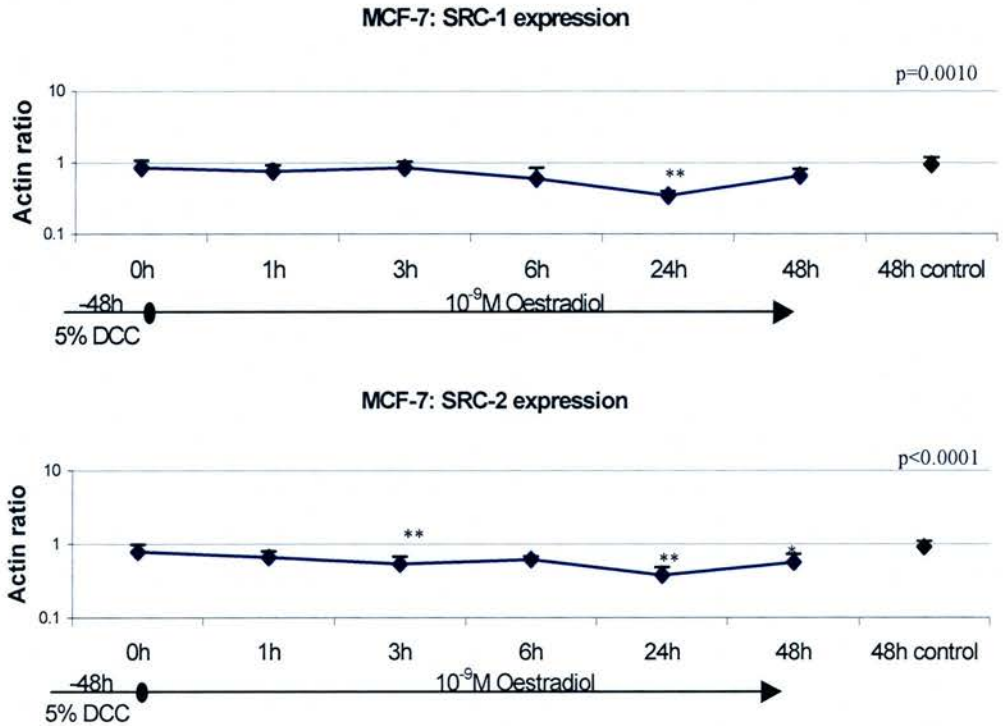


Figure 2.21 A: mRNA expression of SRC-1 and SRC-2 in MCF-7 cells. Cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2. RNA was collected at 0h (72h after plating before treatment), 1h, 3h, 6h, 24h and 48h. A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=$p<0.05$, **=$p<0.01$.

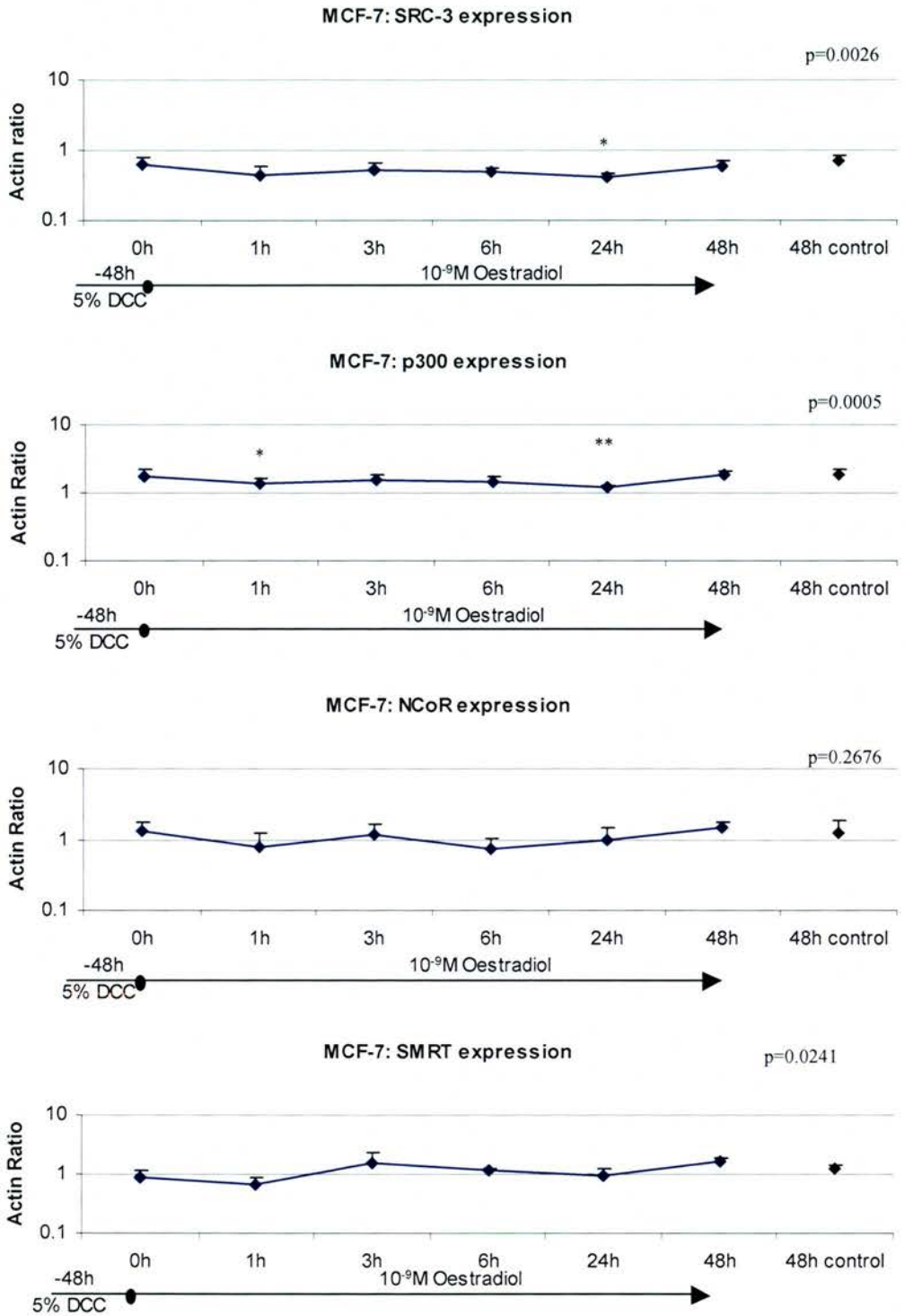


Figure 2.21 B: mRNA expression of SRC-3, p300, NCoR and SMRT in MCF-7 cells. Cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2. RNA was collected at 0h (72h after plating before treatment), 1h, 3h, 6h, 24h and 48h. A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=p<0.05, **=p<0.01.

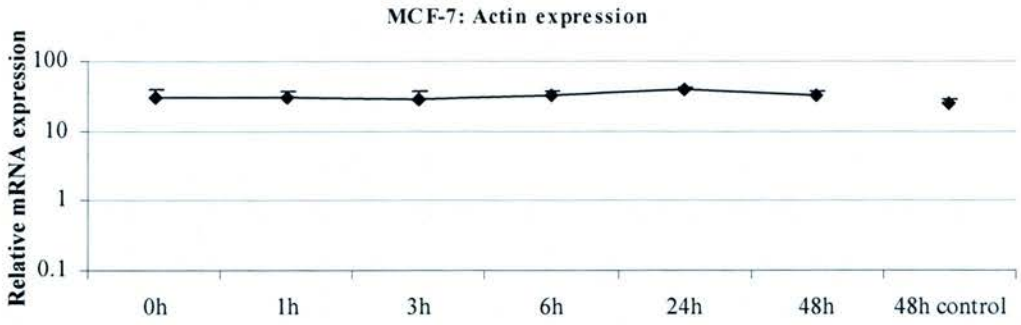


Figure 2.21 C: mRNA expression of actin as a control in MCF-7 cells. Cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2. RNA was collected at 0h (72h after plating before treatment), 1h, 3h, 6h, 24h and 48h. A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=$p<0.05$, **=$p<0.01$.

(ii) Effect of E_2 and tamoxifen on cofactor mRNA and protein expression in MCF-7 cells

As seen in the previous experiment, MCF-7 cells showed similar expression levels for all cofactors (figure 2.22 A and B). Oestrogen showed small stimulatory effects on SRC-1 (3.1 fold), SRC-2 (2.3 fold), SRC-3 (2.4 fold, not statistically significant) and p300 (1.9 fold) expression at 48h when compared to the control of each time group. Not all of these changes were apparent in the previous experiment and some may be due to differences in basal levels for the control groups. Tamoxifen was able to reduce expression levels significantly in SRC-1 and SRC-2 at 6h (1.8 fold and 1.7 fold, respectively). This down-regulation for SRC-1 and SRC-2 was also observed when E_2 is combined with tamoxifen (1.7 fold and 1.5 fold, respectively). A small induction by E_2 was found present at 6h in NCoR as seen before in the E_2 time course.

Unique patterns of expression were seen for mRNA expression of RIP140 and NCoR. Treatment with E_2 produced an induction of RIP140 and NCoR at 6h (4.6 fold and 1.4 fold respectively) and 48h (13.3 fold and 3.8 fold, respectively). Tamoxifen by itself had no effect at 6h and led to a small increase by 48h (2.5 fold and 2.4 fold). For both, RIP140 and NCoR, tamoxifen reversed the effect of E_2 fully at 6h and partially at 48h when both agents were combined.

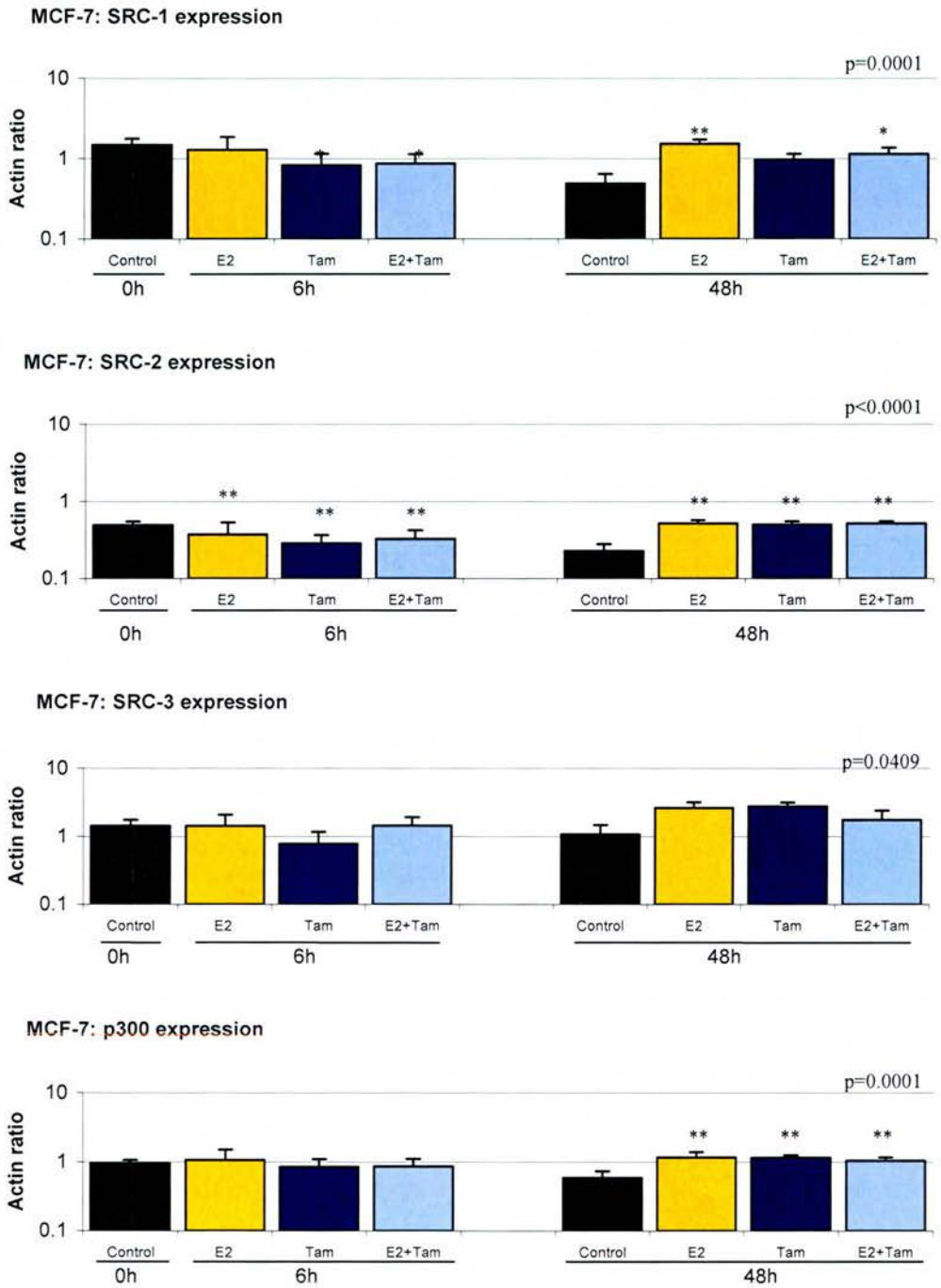


Figure 2.22 A: SRC-1/2/3 and p300 mRNA expression in MCF-7 cells. Cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment of 10^{-9}M E_2, 10^{-6}M tam or 10^{-9}M E_2 and 10^{-6}M tam. The control group was left untreated. RNA was collected at 0h (72h after plating and before treatment), 6h and 48h. Representative experiment is shown of at least two experiments carried out. Each column represents mean of quadruplicate PCR analysis for each sample demonstrating mRNA expression relative to actin expression. Error bars=STD. P-value for variance between all groups determined by one-way ANOVA and multiple comparison Tukey test. Statistical significance noted for treatment groups compared to matched control where *=$p<0.05$, **=$p<0.01$.

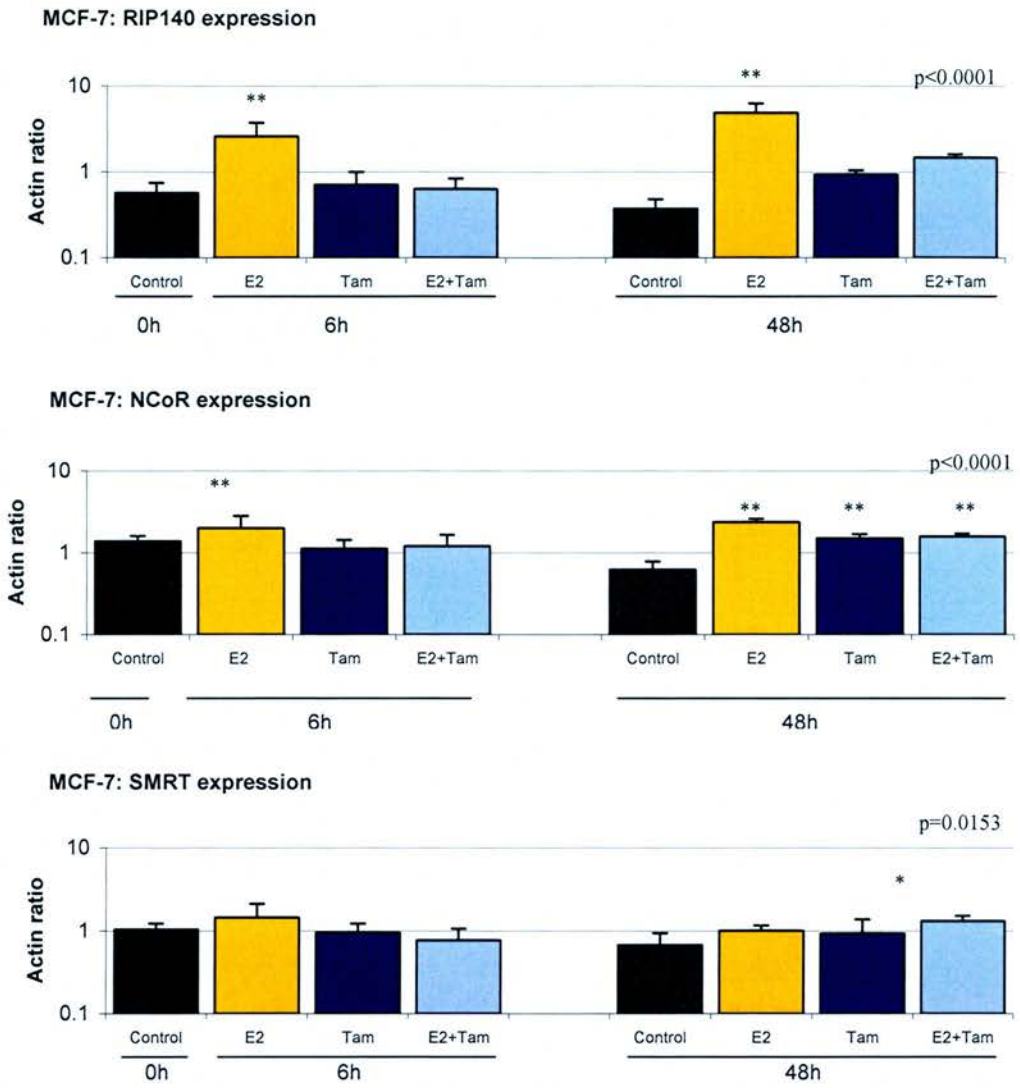


Figure 2.22 B: RIP140, NCoR and SMRT mRNA expression in MCF-7 cells. Cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment of 10^{-9}M E_2, 10^{-6}M tam or 10^{-9}M E_2 and 10^{-6}M tam. The control group was left untreated. RNA was collected at 0h (72h after plating and before treatment), 6h and 48h. Representative experiment is shown of at least two experiments carried out. Each column represents mean of quadruplicate PCR analysis for each sample demonstrating mRNA expression relative to actin expression. Error bars=STD. P-value for variance between all groups determined by one-way ANOVA and multiple comparison Tukey test. Statistical significance noted for treatment groups compared to matched control where *=$p<0.05$, **=$p<0.01$.

When protein levels were analysed, bands corresponding to SRC-2/3, RIP140 and REA were detected in the control groups (figure 2.23). Neither oestrogen nor tamoxifen has any effect on protein expression for any of these cofactors although it has to be noted that RIP140 expression did reveal differences in band numbers for each treatment group. While the control lane showed two or possibly three bands spaced apart, samples treated with E_2 and E_2 +tamoxifen produced uniform double bands. Tamoxifen treatment by itself led to a multiple band cluster representing protein of about 140kDa. SRC-1 protein expression was not detected in control or E_2 treatment but did show faint products in both tamoxifen groups. Aside from SRC-1, the protein expression broadly corresponds to mRNA expression detected in the previous experiment. The amplified protein expression observed in SRC-3 (and SRC-2) compared to SRC-1 confirms published results (Azorsa, D.O. et al. 2001 among others).

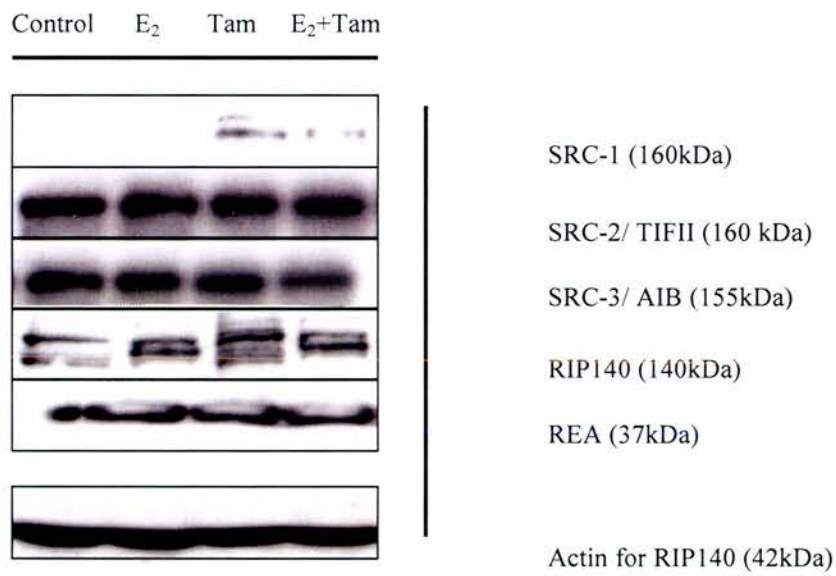


Figure 2.23: Western blot analysis of several cofactors in MCF-7 cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2, 10^{-6}M tamoxifen or 10^{-9}M E_2 and 10^{-6}M tamoxifen. The control group was left untreated. Protein was collected at 48h of treatment. 100µg protein was loaded per lane and detected using anti- SRC-1 (Upstate), SRC-2 (BD Biosciences), SRC-3 (Affinity Bioreagents), RIP140 (Affinity Bioreagents) and REA (Upstate). Total actin was detected using anti – β-actin (CALBIOCHEM®) in all cell lines (one representative cell line shown)as a western blot loading control.

2.4.3 Modulation of coactivators and corepressors in LCC-1 cells

(i) Effect of E_2 on cofactor mRNA expression in LCC-1

Cofactor mRNA expression in LCC-1 cells revealed cell line specific expression patterns (figure 2.24 A -C). Most remarkable was an initial E_2 stimulation at 1h of varying degree for the studied coactivators and corepressors. Increases were about 2-3 fold compared to 0h (highest increase: NCoR 6.3 fold; lowest increase: SRC-3 1.4 fold, although statistically insignificant). Expression generally returned to basal levels by 6h. Basal expression levels do not greatly differ between cofactors. The lowest mRNA expression was observed for SRC-3, the cofactor that also showed the most subtle E_2 stimulation.

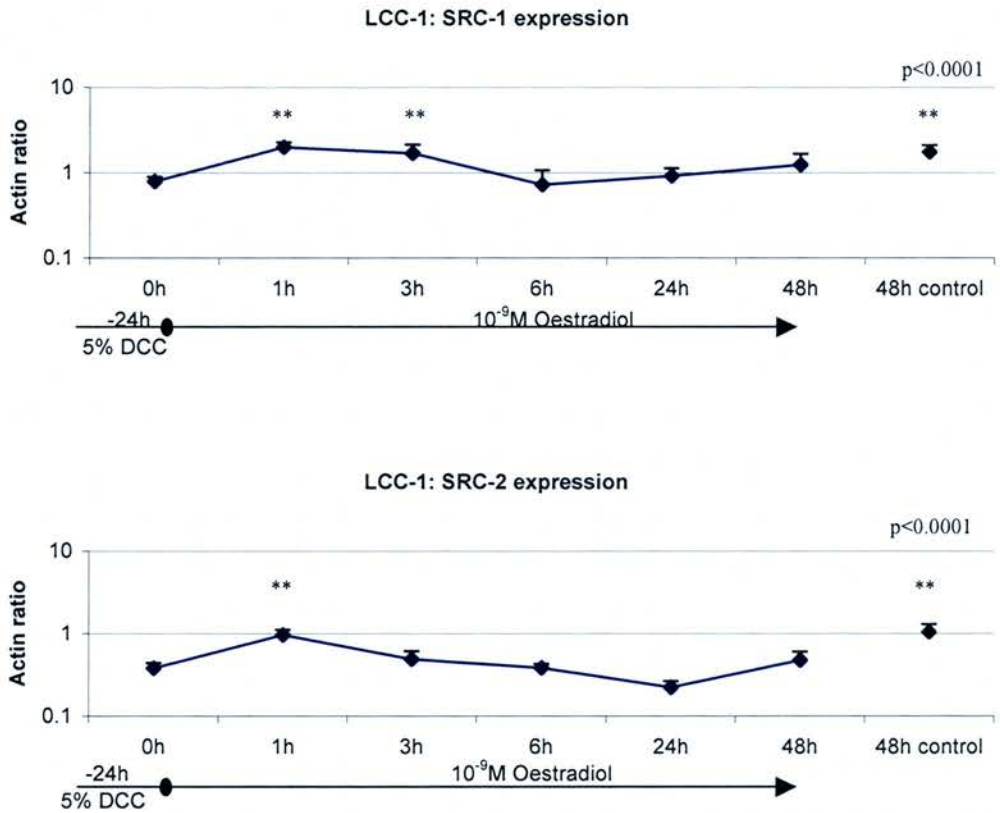


Figure 2.24 A: mRNA expression of SRC-1 and SRC-2 in LCC-1 cells. Cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2. RNA was collected at 0h (72h after plating before treatment), 1h, 3h, 6h, 24h and 48h. A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=$p<0.05$, **=$p<0.01$.

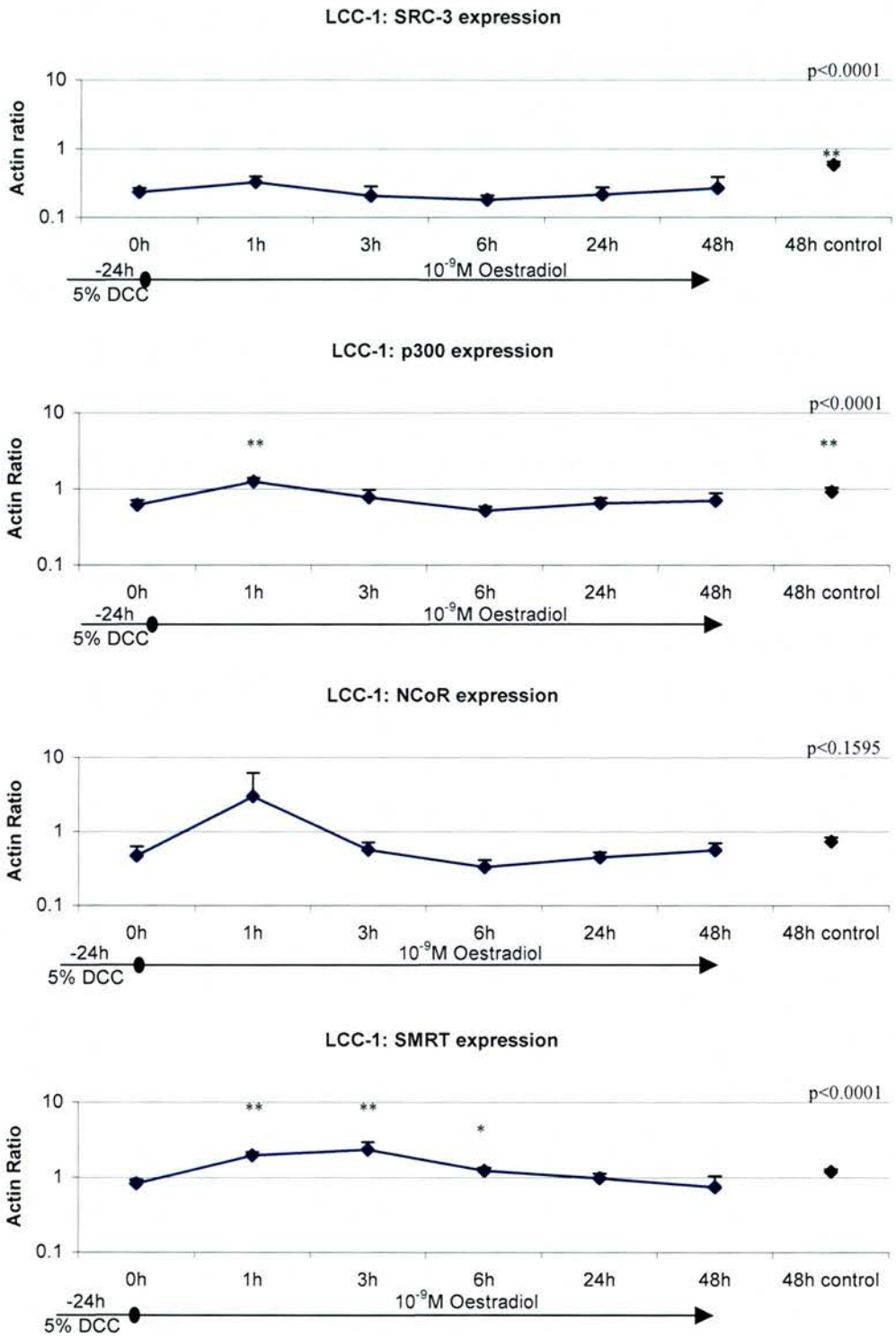


Figure 2.24 B: mRNA expression of SRC-3, p300, NCoR amd SMRT in LCC-1 cells. Cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2. RNA was collected at 0h (72h after plating before treatment), 1h, 3h, 6h, 24h and 48h. A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=$p<0.05$, **=$p<0.01$.

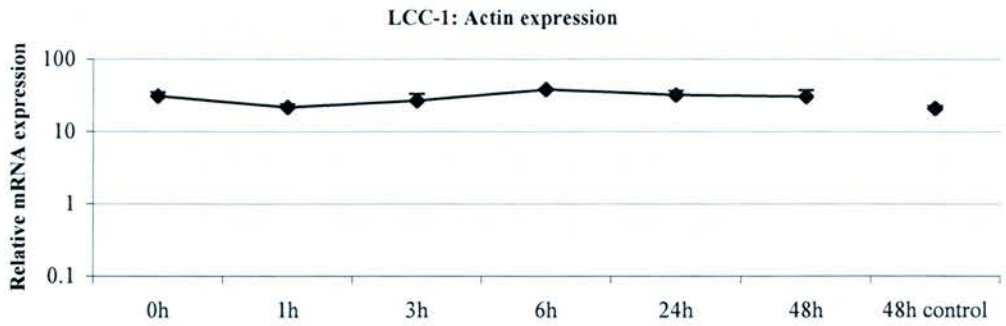


Figure 2.24 C: mRNA expression of actin as control in LCC-1 cells. Cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2. RNA was collected at 0h (72h after plating before treatment), 1h, 3h, 6h, 24h and 48h. A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=$p<0.05$, **=$p<0.01$.

(ii) Effect of E_2 and tamoxifen on cofactor mRNA and protein expression

Comparison between mRNA expression treated with E_2 and tamoxifen revealed only minor differences in LCC-1 cells at 6h and 48h (figure 2.25 A and B). There was no stimulatory effect of E_2 at 48h reflecting observation of the oestrogen time course in the previous section where expression had returned to basal levels by that time. Not observed before was a significant decrease in expression of SRC-3 by E_2 at 6h (7.7 fold) suggesting oestrogen is effectively capable of blocking SRC-3 expression. This E_2 induced decrease was completely overturned by the addition of tamoxifen and expression appeared at basal levels. The oestrogenic effect was lost at 48h. Tamoxifen by itself had no effect at either 6h or 48h.

As observed in MCF-7 cells, RIP140 mRNA expression revealed interesting differences with respect to E_2 and tamoxifen treatment. A comparably high basal expression was observed. At 6h, tamoxifen slightly reduced expression (1.2 fold). The addition of E_2 lead to a further reduction (now 1.7 fold compared to control). In contrast, at 48h tamoxifen dramatically increased mRNA expression (4.3 fold) and the addition of E_2 reduced this effect slightly (3.7 fold compared to control).

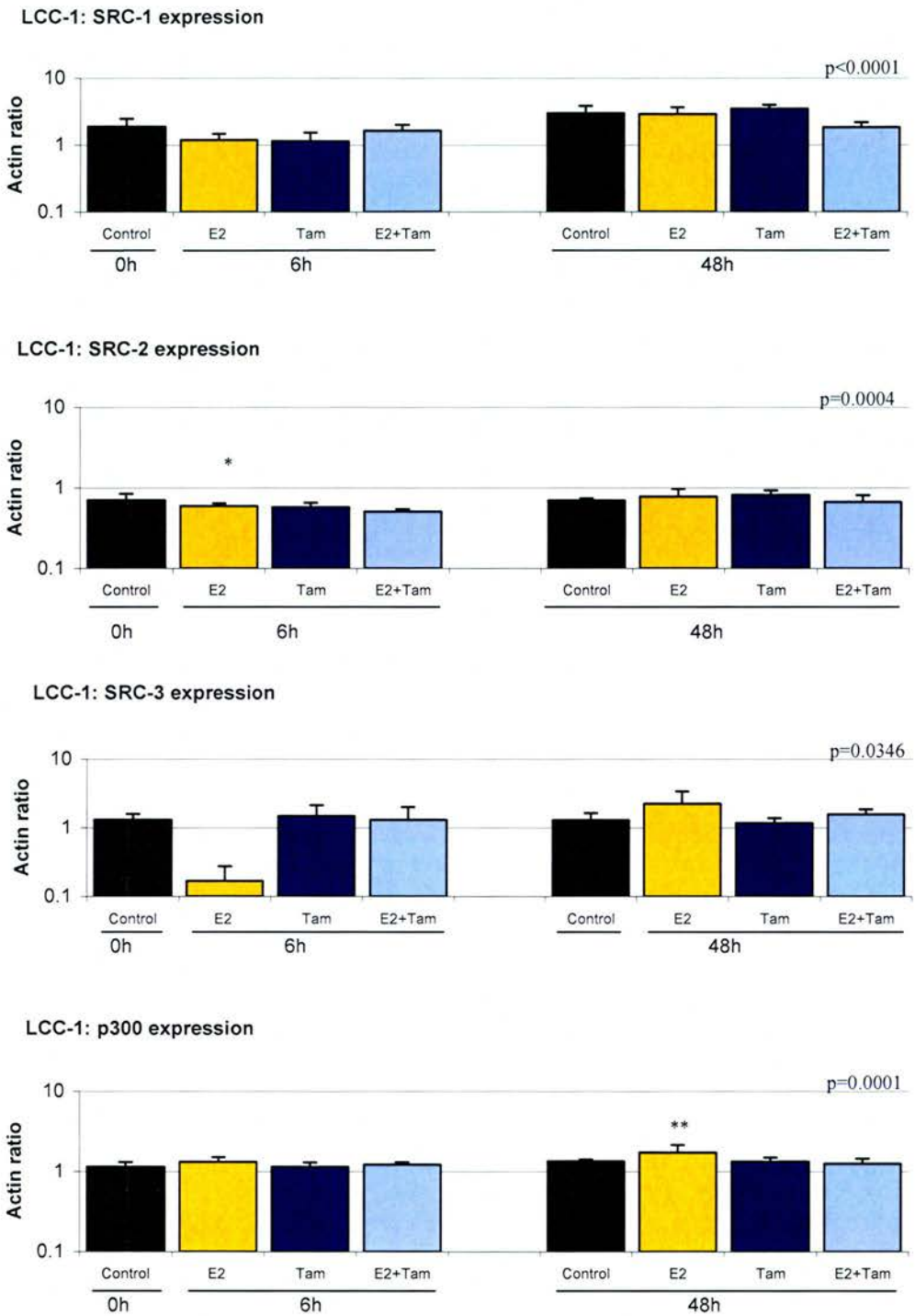


Figure 2.25 A: Coactivator mRNA expression in LCC-1 cells. Cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2, 10^{-6}M tam or 10^{-9}M E_2 and 10^{-6}M tam. The control group was left untreated. RNA was collected at 6h and 48h. Representitive experiment is shown of at least two experiments carried out. Each column represents mean of quadruplet PCR analysis for each sample demonstrating mRNA expression relative to actin expression. Error bars=STD. P-value for variance between all groups determined by one-way ANOVA and multiple comparison Tukey test. Statistical significance noted for treatment groups compared to matched control where *=p<0.05, **=p<0.01.

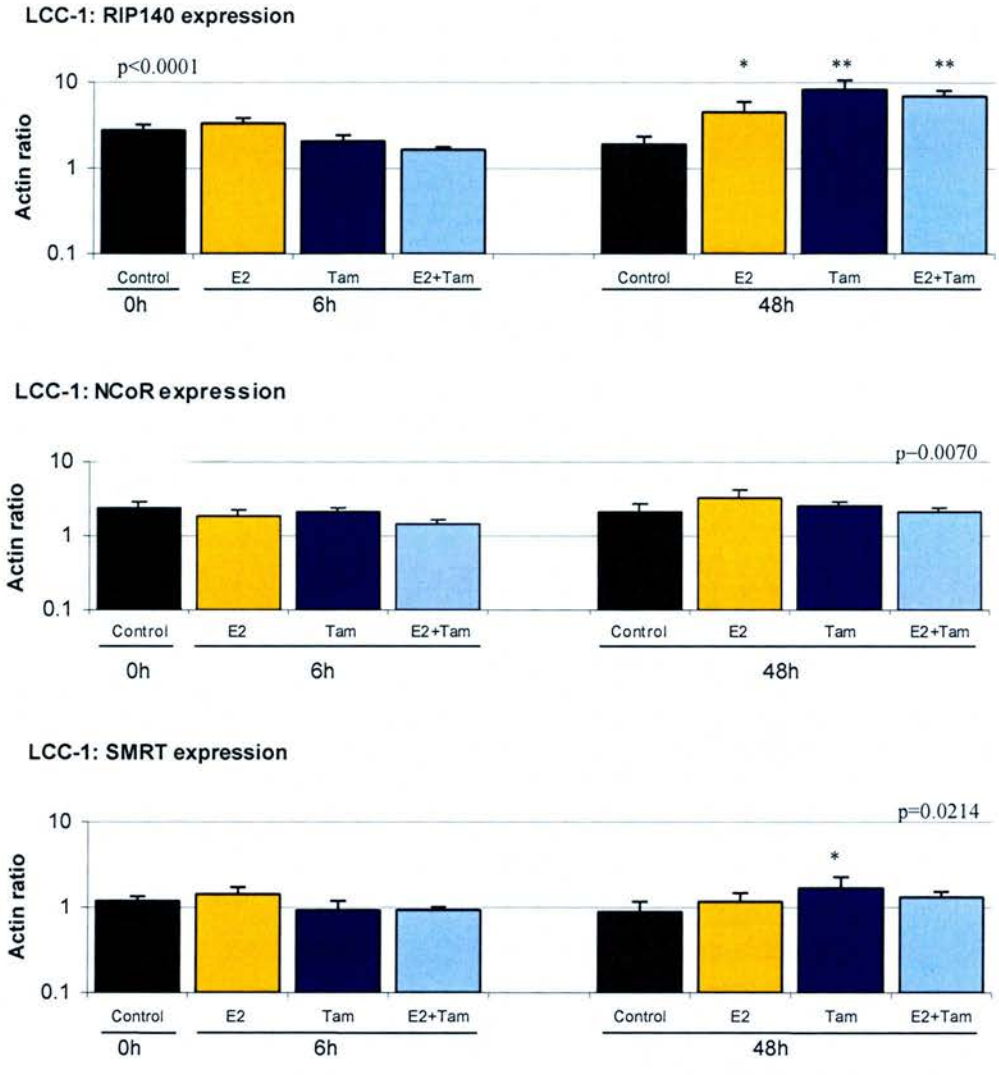


Figure 2.25 B: RIP140 and corepressor mRNA expression in LCC-1 cells. Cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2, 10^{-6}M tam or 10^{-9}M E_2 and 10^{-6}M tam. The control group was left untreated. RNA was collected at 6h and 48h. Representitive experiment is shown of at least two experiments carried out. Each column represents mean of quadruplet PCR analysis for each sample demonstrating mRNA expression relative to actin expression. Error bars=STD. P-value for variance between all groups determined by one-way ANOVA and multiple comparison Tukey test. Statistical significance noted for treatment groups compared to matched control where *=p<0.05, **=p<0.01.

Analysis of protein expression in LCC-1 cells with the same parameters detected a small increase in expression of SRC-1 in the presence of E_2 and tamoxifen (figure 2.26). Roughly even double bands of about 160kDa were observed. This effect had not been seen at the mRNA level. Protein expression appeared at basal level when E_2 and tamoxifen are combined. An increase in expression was also observed for RIP140 in the presence of E_2. Here, particularly the smaller protein of the double band observed appeared stronger with E_2. SRC-2, SRC-3 and REA were readily detected but did not show any obvious expression differences with respect to the different treatment groups.

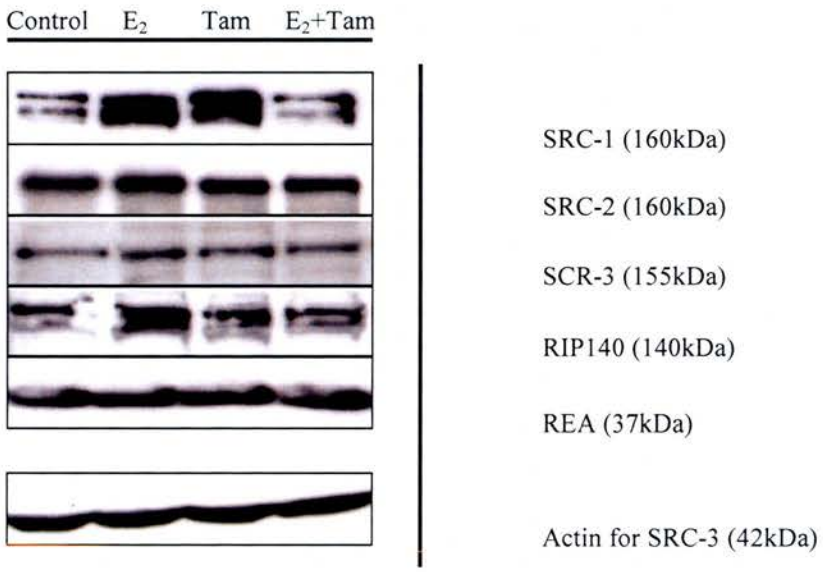


Figure 2.26: Western blot analysis of several cofactors in LCC-1 cells were seeded in reduced media conditions for 24h before treatment with 10^{-9}M E_2, 10^{-6}M tam or 10^{-9}M E_2 and 10^{-6}M tam. The control group was left untreated. Protein was collected at 48h of treatment. 100µg protein was loaded per lane and detected using anti- SRC-1 (Upstate), SRC-2 (BD Biosciences), SRC-3 (Affinity Bioreagents), RIP140 (Affinity Bioreagents) and REA (Upstate) Total actin was detected using anti – β-actin (CALBIOCHEM®) in all cell lines (one representative cell line shown)as a western blot loading control.

2.4.4 Modulation of coactivators and corepressors in LCC-2 cells

(i) Effect of E_2 on cofactor mRNA expression

The most apparent expression characteristic in LCC-2 cells was a common down-regulation at 3h of all cofactors with the exception of SMRT (figure 2.27 A- C). Down-regulation was gradual and ranged from 2.4 fold in p300 to 4 fold in SRC-3. Expression increased steadily again after 3h and reached basal levels by 48h. SMRT mRNA expression differed from the other cofactors to such effect that E_2 continuously induced expression up to 6h (2.5 fold). mRNA levels were reduced thereafter.

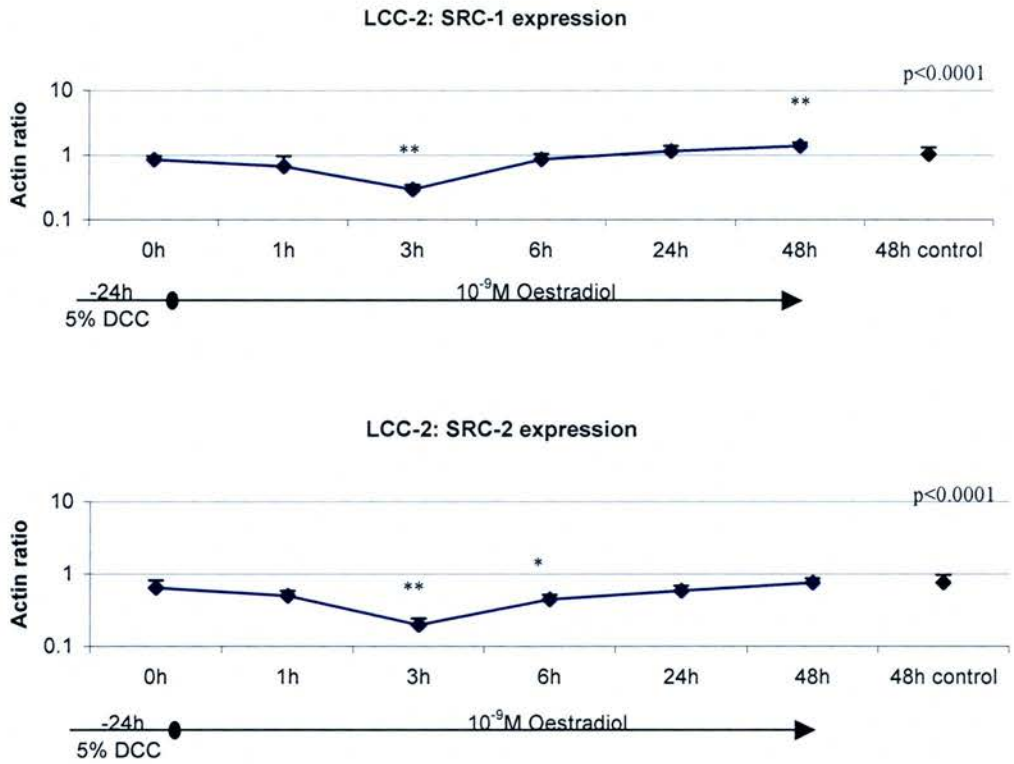


Figure 2.27 A: mRNA expression of SRC-1 and SRC-2 in LCC-2 cells. Cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2. RNA was collected at 0h (72h after plating before treatment), 1h, 3h, 6h, 24h and 48h. A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=$p<0.05$, **=$p<0.01$.

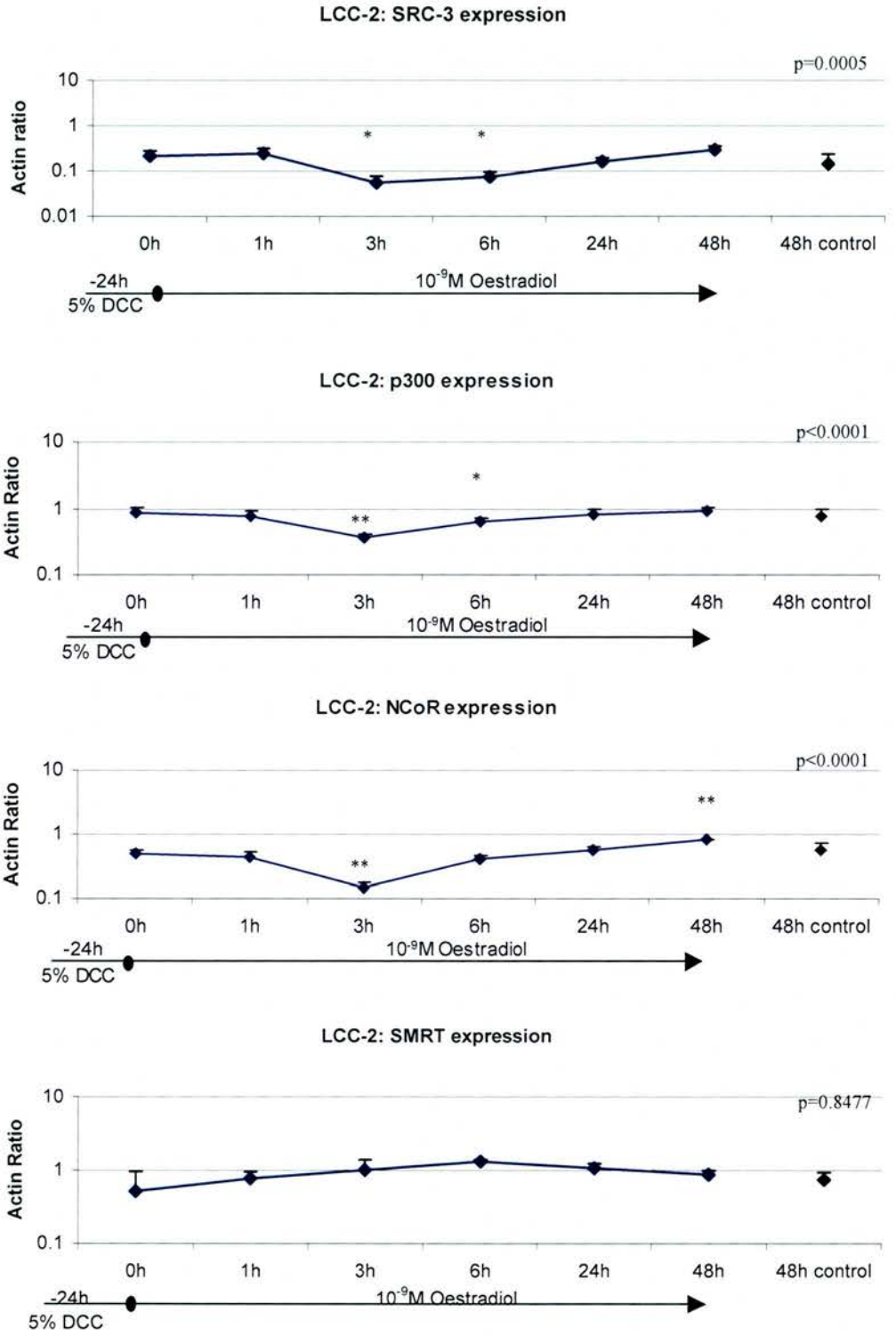


Figure 2.27 B: mRNA expression of SRC-3, p300, NCoR and SMRT in LCC-2 cells. Cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2. RNA was collected at 0h (72h after plating before treatment), 1h, 3h, 6h, 24h and 48h. A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=$p<0.05$, **=$p<0.01$.

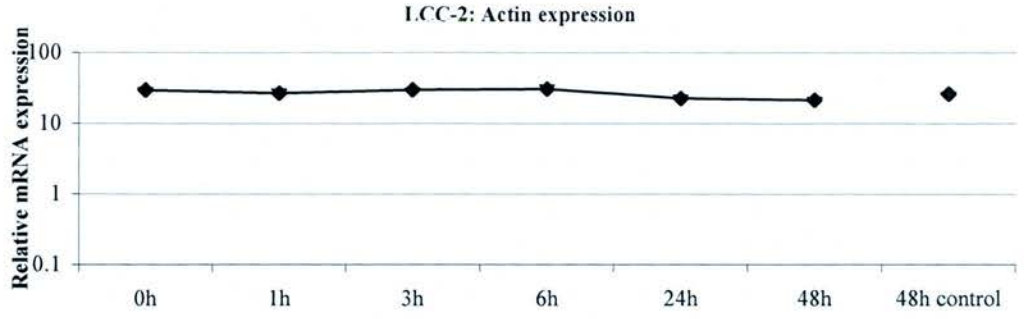


Figure 2.27 C: mRNA expression of actin in LCC-2 cells. Cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2. RNA was collected at 0h (72h after plating before treatment), 1h, 3h, 6h, 24h and 48h. A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=$p<0.05$, **=$p<0.01$.

(ii) Effect of E_2 and tamoxifen on cofactor mRNA and protein expression

The oestrogenic modulation observed in the previous experiment were confirmed in this part of the analysis looking at the effects of E_2 and tamoxifen (figure 2.28 A and B). Reduced expression levels were still apparent at 6h for SRC-1, SRC-2 and SRC-3 (1.8 fold, 3-fold, 3.1 fold, respectively). At 48h, mRNA levels were largely unaffected by E_2 or tamoxifen. The most significant changes were again identified for RIP140 and here particularly at 48h. E_2 had a stimulatory effect on mRNA expression (2.8 fold). Tamoxifen as well as tamoxifen plus E_2 also increased RIP140 expression but even more strongly than E_2 (3.5 fold and 4.3 fold, respectively). This observation had also been made in LCC-1 cells (figure 2.25). The expression of both corepressors mRNA, NCoR and SMRT, was largely unaffected by the addition of oestrogen or tamoxifen.

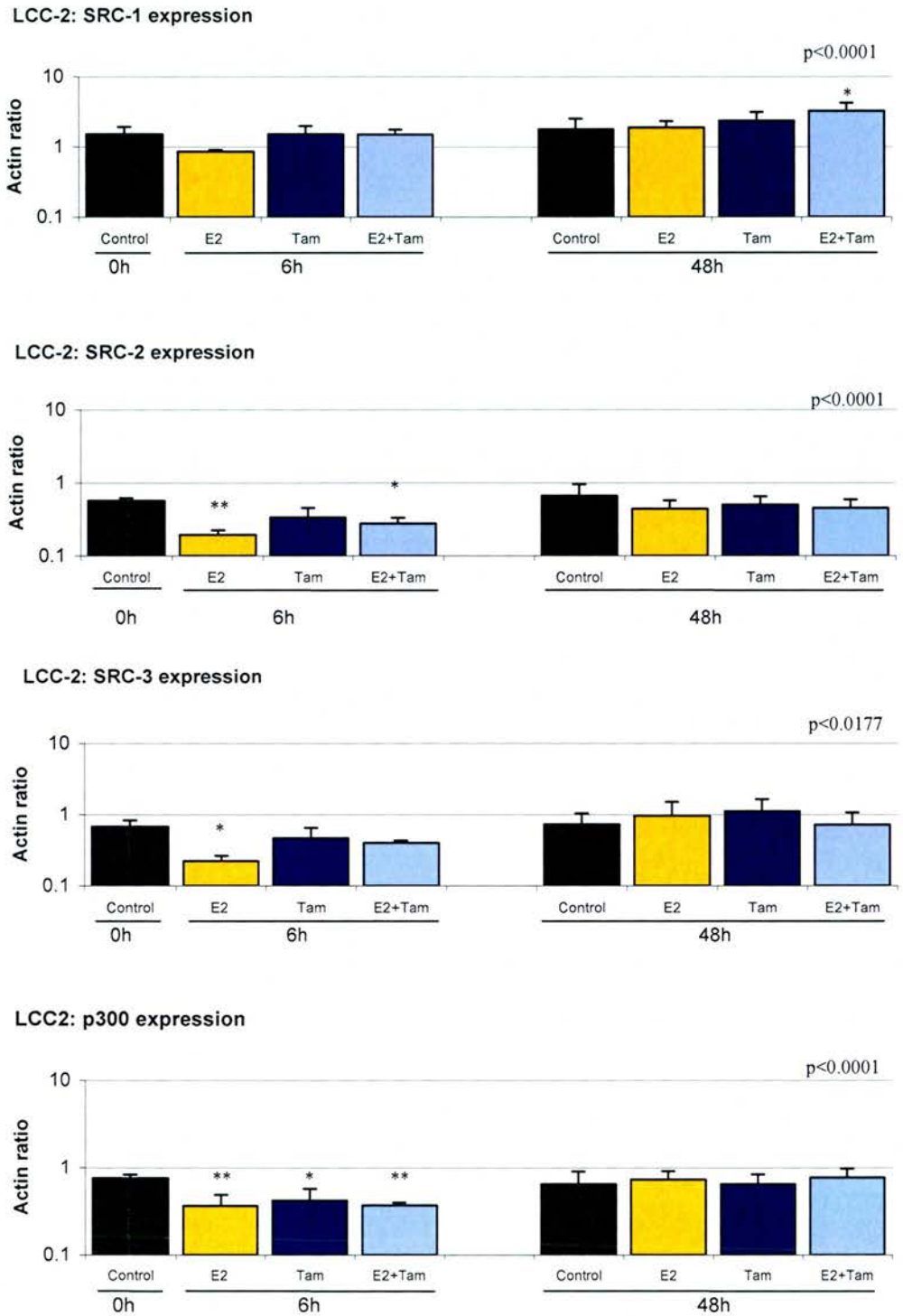


Figure 2.28 A: p160 and p300 mRNA expression in LCC-2 cells. Cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2, 10^{-6}M tam or 10^{-9}M E_2 and 10^{-6}M tam. The control group was left untreated. RNA was collected at 6h and 48h. Representative experiment is shown of at least two experiments carried out. Each column represents mean of quadruplicate PCR analysis for each sample demonstrating mRNA expression relative to actin expression. Error bars=STD. P-value for variance between all groups determined by one-way ANOVA and multiple comparison Tukey test. Statistical significance noted for treatment groups compared to matched control where *=p<0.05, **=p<0.01.

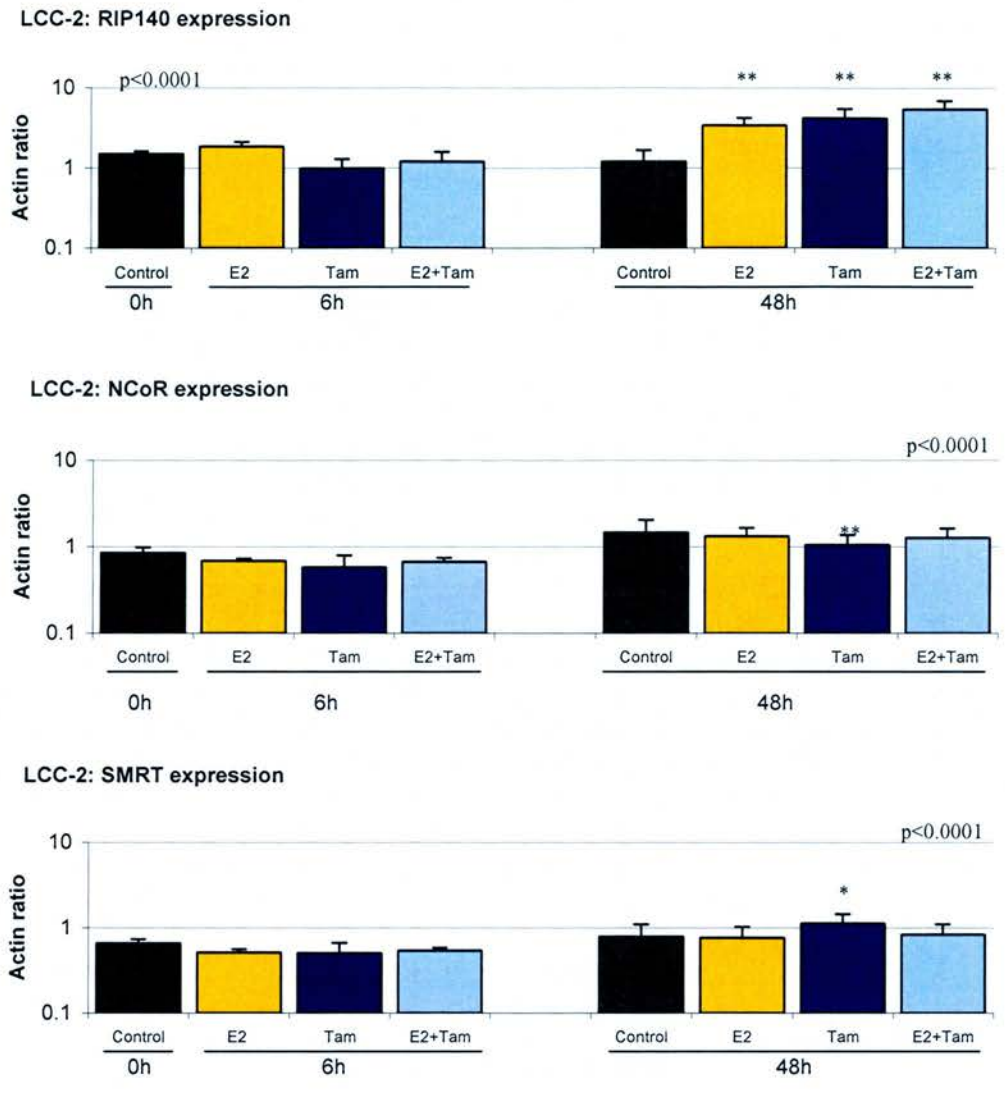


Figure 2.28 B: RIP140 and corepressor mRNA expression in LCC-2 cells. Cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2, 10^{-6}M tam or 10^{-9}M E_2 and 10^{-6}M tam. The control group was left untreated. RNA was collected at 6h and 48h. Representative experiment is shown of at least two experiments carried out. Each column represents mean of quadruplicate PCR analysis for each sample demonstrating mRNA expression relative to actin expression. Error bars=STD. P-value for variance between all groups determined by one-way ANOVA and multiple comparison Tukey test. Statistical significance noted for treatment groups compared to matched control where *=$p<0.05$, **=$p<0.01$.

Protein expression was readily detected for all investigated cofactors in LCC-2 cells (figure 2.29). E_2 had opposing effects within expression of the p160 family proteins in this cell line. While the hormone increased expression of SRC-1 and SRC-3, a clear decrease was observed in SRC-2 expression. Remarkable differences were observed in band numbers in SRC-1 and RIP140 protein expression. Both factors were detected as strong single bands in the control group and in the presence of tamoxifen as opposed to strong double bands in the presence of E_2 and E_2 plus tamoxifen. This trend had been observed in LCC-1 cells (figure 2.26).

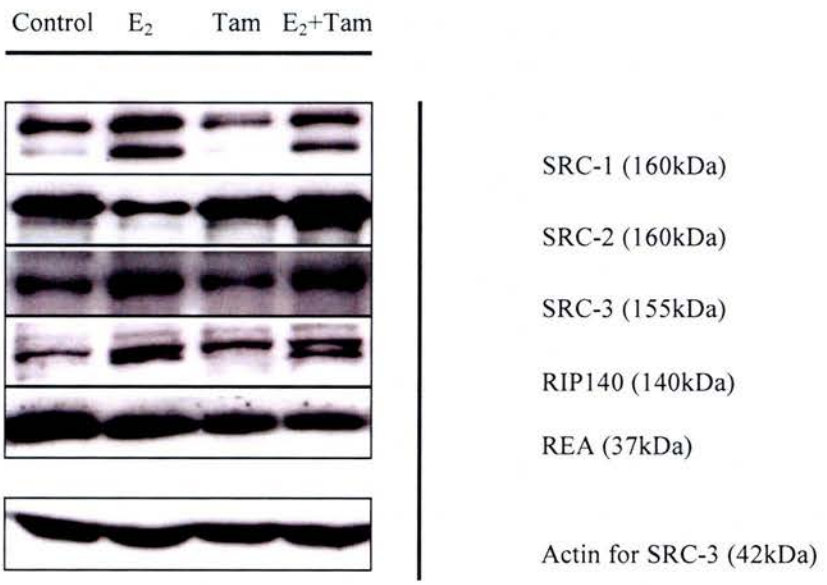


Figure 2.29: Western blot analysis of several cofactors in LCC-2 cells placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2, 10^{-6}M tam or 10^{-9}M E_2 and 10^{-6}M tam. The control group was left untreated. Protein was collected at 48h of treatment. 100µg protein was loaded per lane and detected using anti- SRC-1 (Upstate), SRC-2 (BD Biosciences), SRC-3 (Affinity Bioreagents), RIP140 (Affinity Bioreagents) and REA (Upstate). Total actin was detected using anti – β-actin (CALBIOCHEM®) in all cell lines (one representative cell line shown)as a western blot loading control.

2.4.5 Modulation of coactivators and corepressors in LCC-9 cells

(i) Effect of E_2 on cofactor mRNA expression

In LCC-9 cells, oestrogen reduced most of the investigated cofactors similar to the observations in LCC-2 cells (figure 2.30 A- C). Between 0h and 6h, the SRC family, p300 and NCoR mRNA were down-regulated to varying degrees. Most strongly reduced was NCoR gene expression at 6h (3.4 fold) but also p300 (2.1 fold) and SRC-2 (2.9 fold). The expression levels recovered to basal levels between 24h and 48h. The exception was once again found to be SMRT mRNA which remained largely unchanged by the addition of oestrogen.

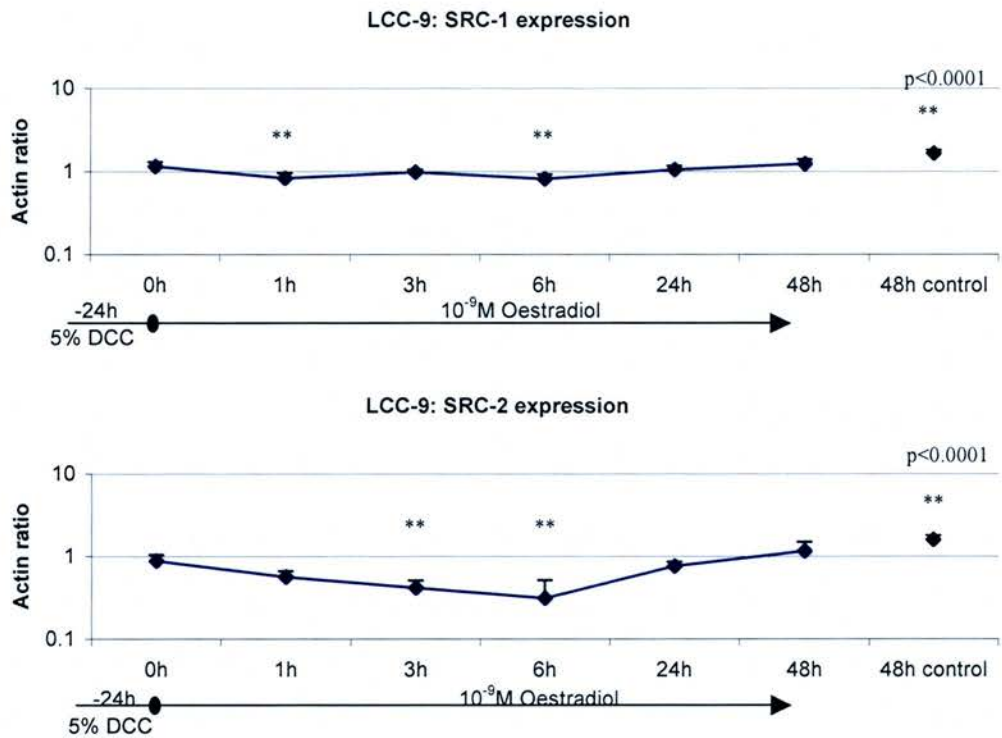


Figure 2.30 A: mRNA expression of SRC-1 and SRC-2 in LCC-9 cells. Cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2. RNA was collected at 0h (72h after plating before treatment), 1h, 3h, 6h, 24h and 48h. A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=p<0.05, **=p<0.01.

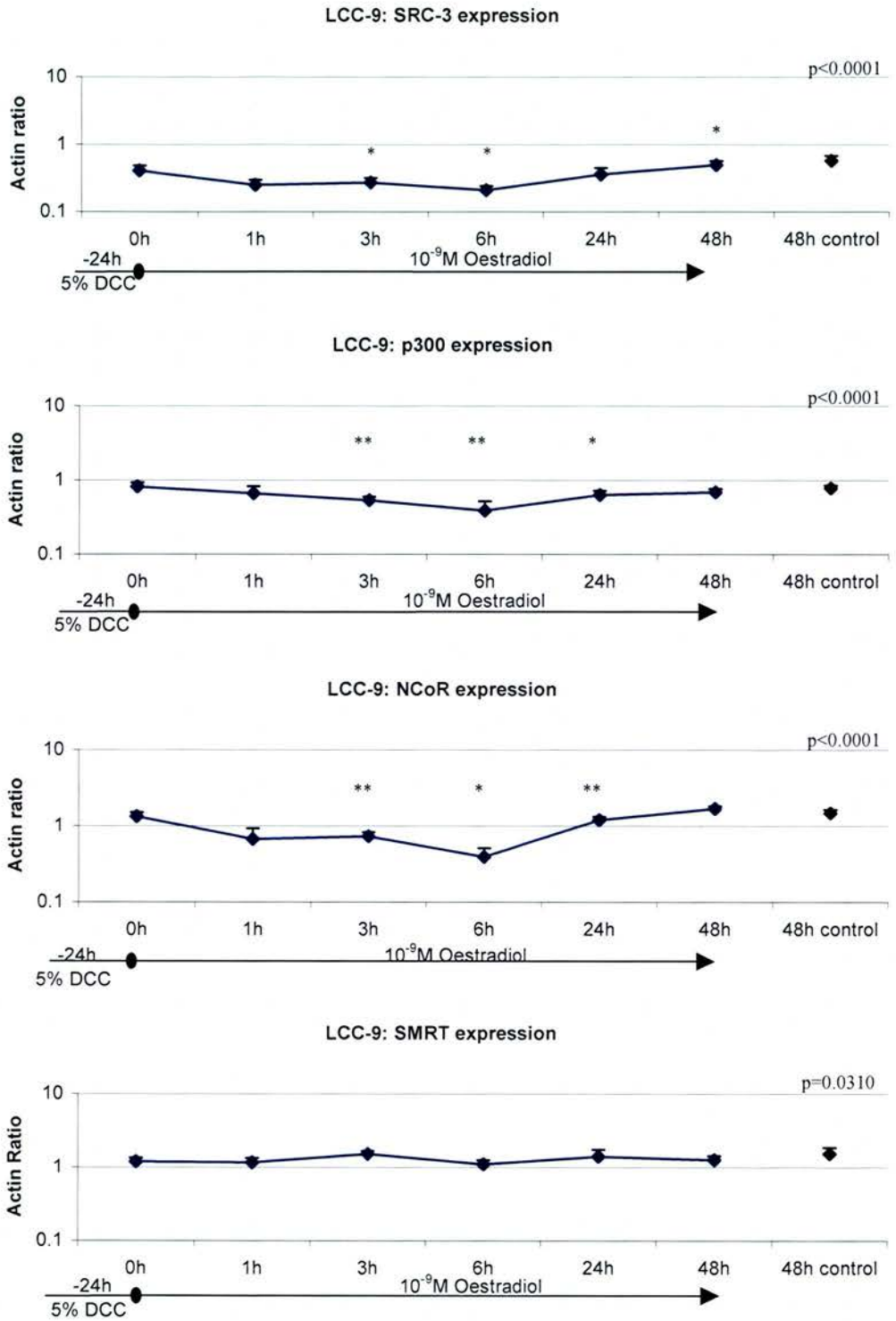


Figure 2.30 B: mRNA expression of RIP140 and corepressors in LCC-9 cells. Cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2. RNA was collected at 0h (72h after plating before treatment), 1h, 3h, 6h, 24h and 48h. A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=$p<0.05$, **=$p<0.01$.

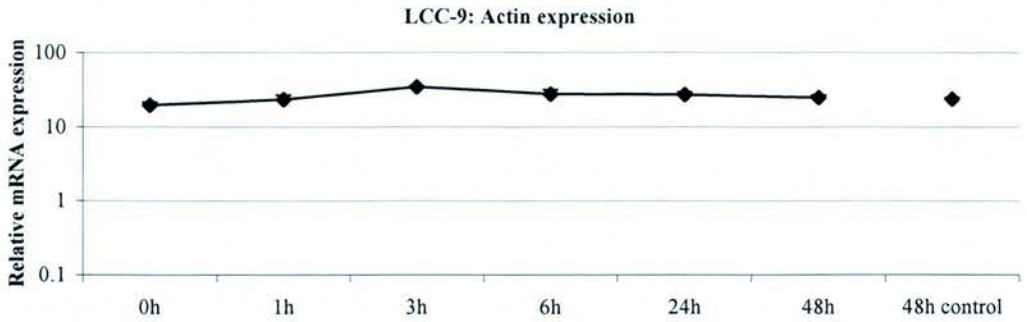


Figure 2.30 C: mRNA expression of actin in LCC-9 cells. Cells were seeded in complete media for 24h before treatment with 10^{-9}M E_2, 10^{-6}M tam or 10^{-9}M E_2 and 10^{-6}M tam. The control group was left untreated. RNA was collected at 0h (72h after plating before treatment), 1h, 3h, 6h, 24h and 48h. Representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Error bars =STD. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnet's multiple comparison test where *=$p<0.05$, **=$p<0.01$.

(ii) Effect of E_2 on cofactor mRNA and protein expression

In general, expression changes in response to oestrogen and tamoxifen were subtle at both time points in LCC-9 cells (figure 2.31 A and B). As mentioned in the previous section, oestrogen only shows small inhibitory effects on gene expression although some trends could not be detected to the same degree again. This is particularly apparent for NCoR where oestrogen does not show a significant reduction as seen in the time course. These differences may be due to cells collected during different functional states in their cell cycle. Small changes in expression were detected in the presence of tamoxifen namely for p300 and NCoR. p300 mRNA and NCoR mRNA were decreased by tamoxifen at the earlier time point (1.8 fold and 2.3 fold, respectively) and by tamoxifen plus E_2 (1.3 fold and 2.1 fold, respectively).

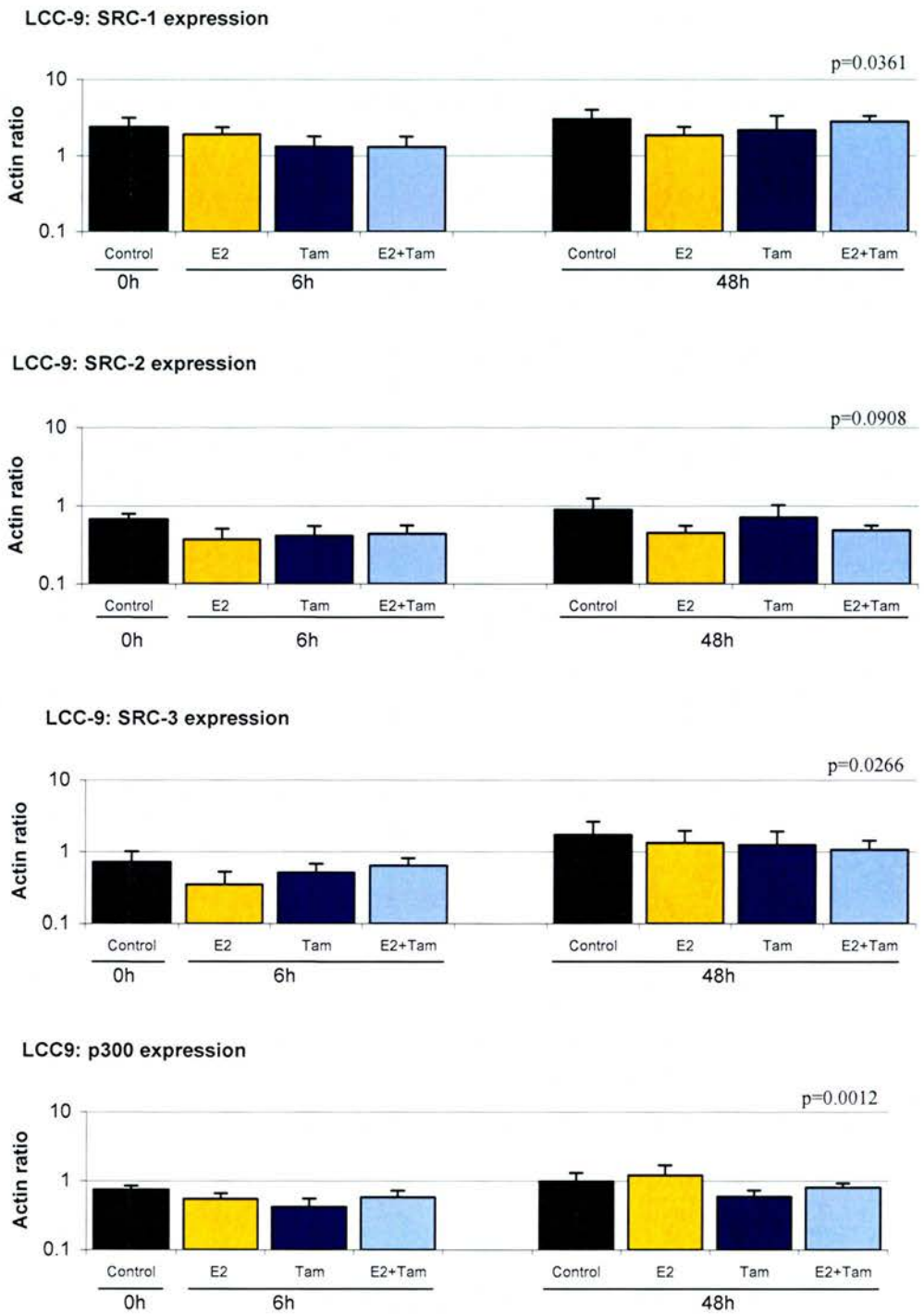


Figure 2.31 A: p160 and p300 mRNA expression in LCC-9 cells. Cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2, 10^{-6}M tam or 10^{-9}M E_2 and 10^{-6}M tam. The control group was left untreated. RNA was collected at 6h and 48h. Representative experiment is shown of at least two experiments carried out. Each column represents mean of quadruplicate PCR analysis for each sample demonstrating mRNA expression relative to actin expression. Error bars=STD. P-value for variance between all groups determined by one-way ANOVA and multiple comparison Tukey test. Statistical significance noted for treatment groups compared to matched control where *=$p<0.05$, **=$p<0.01$.

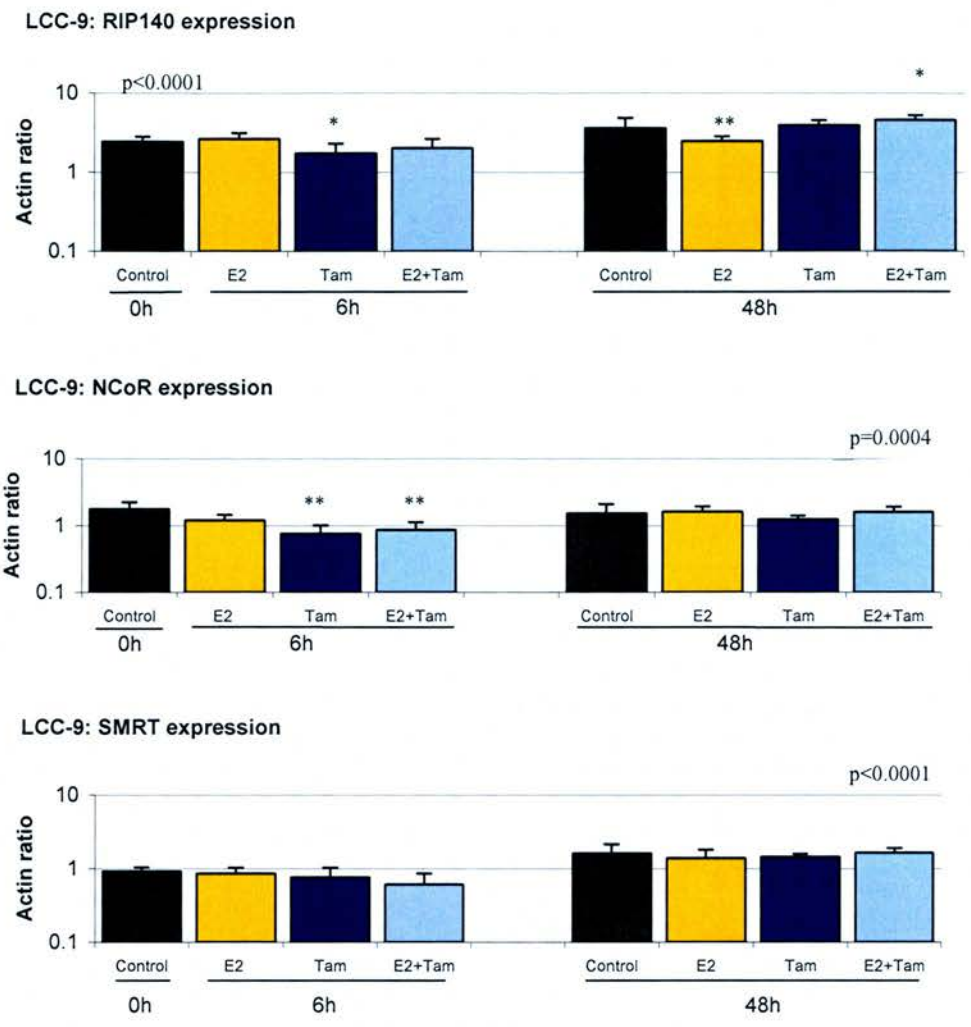


Figure 2.31 B: RIP140 and corepressor mRNA expression in LCC-9 cells. Cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2, 10^{-6}M tam or 10^{-9}M E_2 and 10^{-6}M tam. The control group was left untreated. RNA was collected at 6h and 48h. Representative experiment is shown of at least two experiments carried out. Each column represents mean of quadruplicate PCR analysis for each sample demonstrating mRNA expression relative to actin expression. Error bars=STD. P-value for variance between all groups determined by one-way ANOVA and multiple comparison Tukey test. Statistical significance noted for treatment groups compared to matched control where *=p<0.05, **=p<0.01.

The protein expression for coactivators and corepressors was consistent with mRNA findings with respect to oestrogen and tamoxifen regulation (figure 2.32). Neither agent showed any marked influence on expression. Within the p160 family, SRC-2 protein was particularly strongly present while SRC-1 and SRC-3 were almost undetectable. This observation does not correspond to mRNA findings. Also strongly expressed were REA and RIP140. The latter exhibited multiple bands representing the about 140kDA protein with one distinctly darker band revealed for all treatment groups.

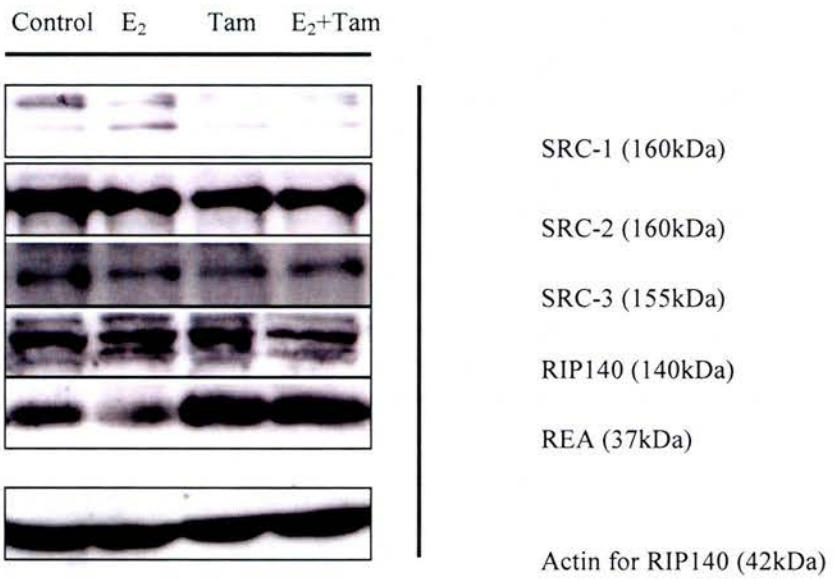


Figure 2.32: Western blot analysis of several cofactors in LCC-9 cells placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2, 10^{-6}M tam or 10^{-9}M E_2 and 10^{-6}M tam. The control group was left untreated. Protein was collected at 48h of treatment. 100µg protein was loaded per lane and detected using anti- SRC-1 (Upstate), SRC-2 (BD Biosciences) and SRC-3 (Affinity Bioreagents), RIP140 (Affinity Bioreagents) and REA (Upstate). Total actin was detected using anti – β-actin (CALBIOCHEM®) in all cell lines (one representative cell line shown)as a western blot loading control.

2.4.6 Modulation of coactivators and corepressors in LY2 cells

(i) Effect of E_2 on cofactor mRNA expression in LY2 cells

Analogous to the effect seen in LCC-9 cells, oestrogen only slightly down-regulated the expression of cofactor mRNA by 6h in LY2 cells. Changes varied between 1.8 fold for NCoR and 1.5 fold for p300. Expression generally tended to recover by 48h. SRC-3 exhibited a particularly low expression. The expression profile for SMRT was again slightly different than the other cofactors. SMRT demonstrated a small down-regulation at 48h (1.6 fold). A limited time course of 6h and 48h only was investigated for LY2 cell (figure 2.33 A and B).

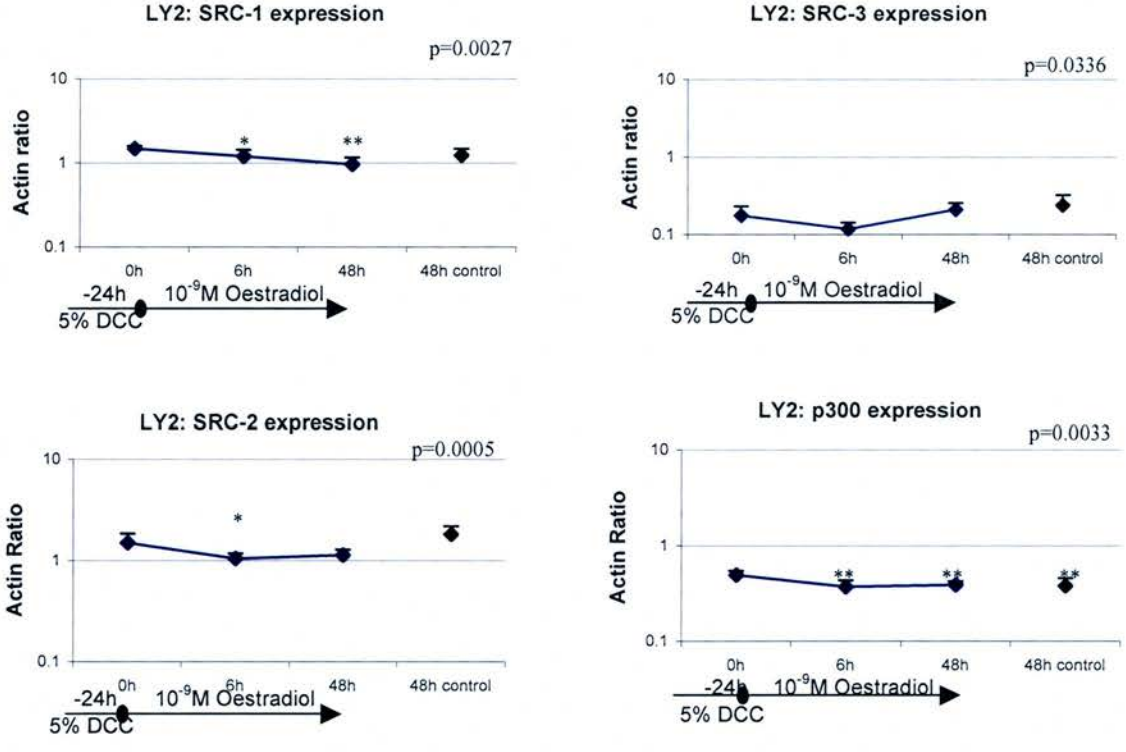


Figure 2.33 A: mRNA expression of p160 cofactors and p300 in LY2 cells. Cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2. RNA was collected at 0h (72h after plating before treatment), 6h and 48h. A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=$p<0.05$, **=$p<0.01$.

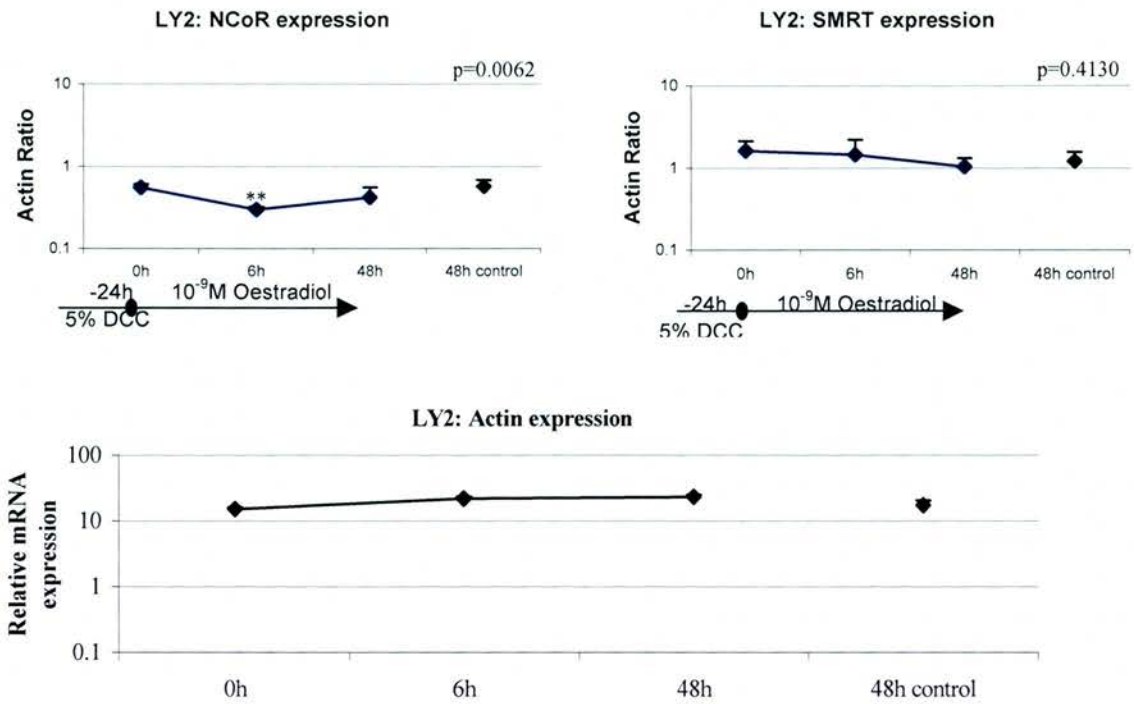


Figure 2.33 B: mRNA expression of corepressors and actin in LY2 cells. Cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2. RNA was collected at 0h (72h after plating before treatment), 6h and 48h. A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=$p<0.05$, **=$p<0.01$.

(ii) Effect of E_2 and tamoxifen on cofactor mRNA and protein expression in LY2 cells

mRNA expression for the p160 family members revealed several changes in response to tamoxifen (figure 2.34 A and B). Noteworthy are significant changes in RIP140, NCoR and SMRT. At 6h, E_2 slightly increased expression for RIP140 (1.3 fold). Tamoxifen reduced the expression (2.1 fold) and not only actively reversed the effect of E_2 but reduced expression below basal levels (1.6 fold). Interestingly, at 48h, E_2 showed a stronger stimulatory effect (2.0 fold) and tamoxifen no longer inhibited but also stimulated gene expression (1.7 fold). The combination of E_2 and tamoxifen led to a 1.99 fold expression increase compared to the matched control. In the structurally similar NCoR and SMRT, E_2 had a small inhibitory effect on mRNA expression at 6h (1.9 fold and 1.3 fold). The same could be observed for tamoxifen (2.4 fold and 1.8 fold, respectively) and E_2 plus tamoxifen (2.6 fold and 1.6 fold). At 48h, all three treatment versions revealed an up-regulatory trend for both NCoR (not

significant) and SMRT (1.1 fold, 1.9 fold and 2.2 fold for E_2, tamoxifen and E_2 plus tamoxifen, respectively).

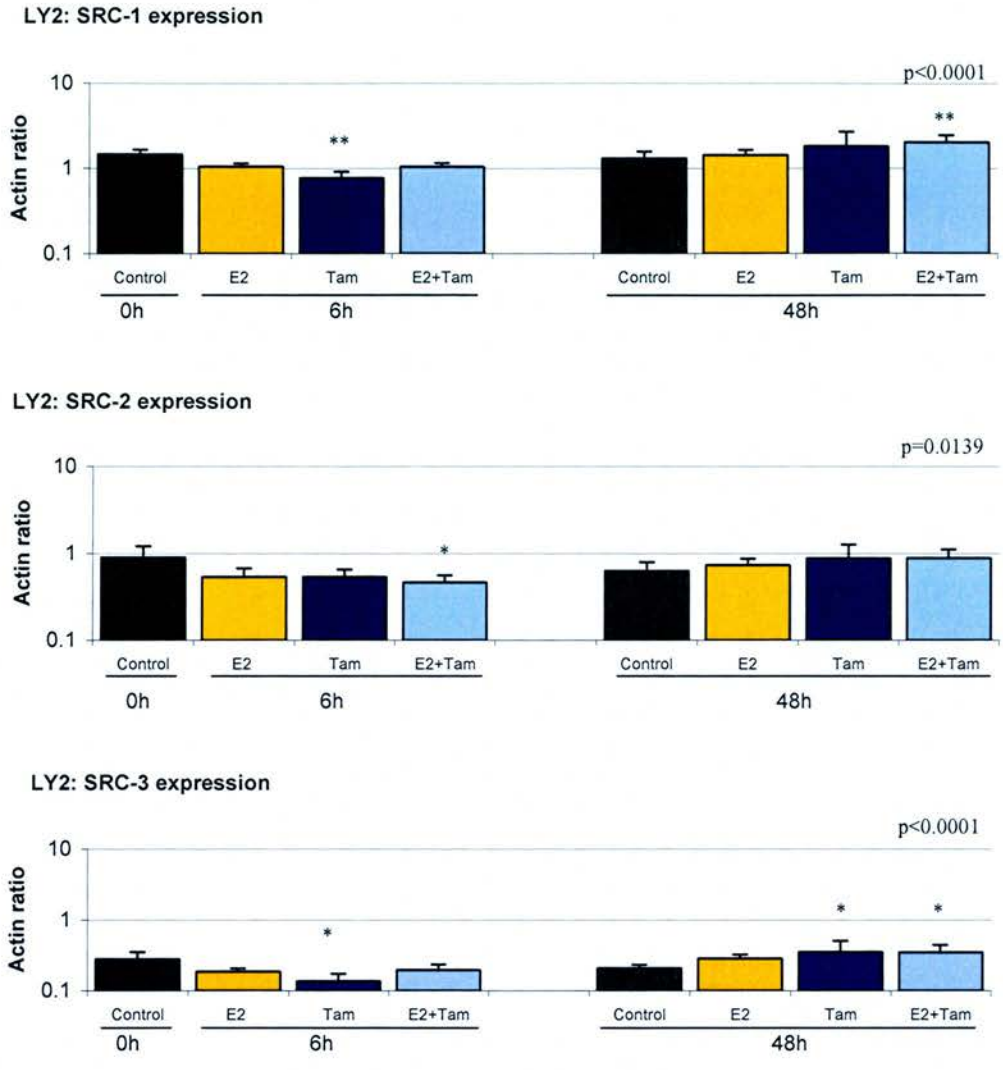


Figure 2.34 A: p160 mRNA expression in LY2 cells. Cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2, 10^{-6}M tam or 10^{-9}M E_2 and 10^{-6}M tam. The control group was left untreated. RNA was collected at 6h and 48h. Representative experiment is shown of at least two experiments carried out. Each column represents mean of quadruplicate PCR analysis for each sample demonstrating mRNA expression relative to actin expression. Error bars=STD. P-value for variance between all groups determined by one-way ANOVA and multiple comparison Tukey test. Statistical significance noted for treatment groups compared to matched control where *=$p<0.05$, **=$p<0.01$

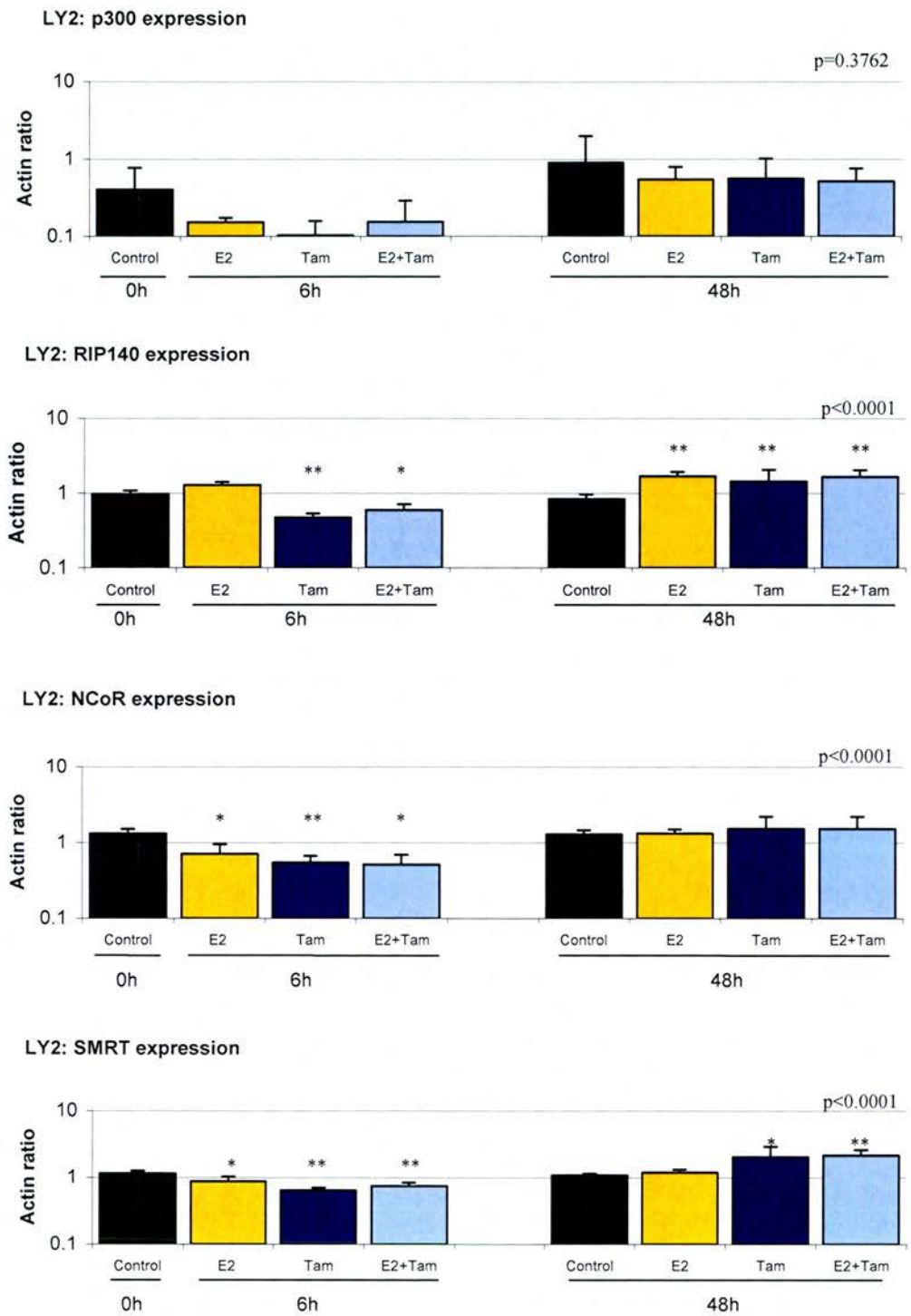


Figure 2.34 B: p300, RIP140, NCoR and SMRT mRNA expression in LY2 cells. Cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2, 10^{-6}M tam or 10^{-9}M E_2 and 10^{-6}M tam. The control group was left untreated. RNA was collected at 6h and 48h. Representative experiment is shown of at least two experiments carried out. Each column represents mean of quadruplicate PCR analysis for each sample demonstrating mRNA expression relative to actin expression. Error bars=STD. P-value for variance between all groups determined by one-way ANOVA and multiple comparison Tukey test. Statistical significance noted for treatment groups compared to matched control where *=$p<0.05$, **=$p<0.01$.

In contrast to the parental MCF-7 cell line and the sublines mentioned so far, protein expression for cofactors was not easily detected in LY2 cells with the exception of SRC-2 (figure 2.35). SRC-2 protein was revealed to be present in all treatment groups and was represented by a sharp band of about 160 kDa. A small down-regulation may be observed with tamoxifen treatment. This was also observed for SRC-1. All other cofactors were detected in the form of faint bands although both RIP140 and SRC-2 revealed characteristic multiple bands as observed previously.

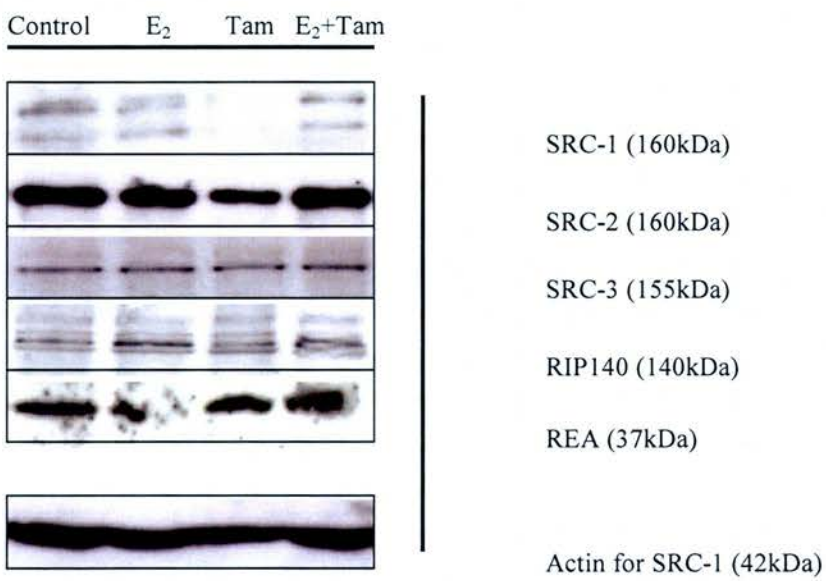


Figure 2.35: Western blot analysis of several cofactors in LY2 cells placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2, 10^{-6}M tam or 10^{-9}M E_2 and 10^{-6}M tam. The control group was left untreated. Protein was collected at 48h of treatment. 100μg protein was loaded per lane and detected using anti- SRC-1 (Upstate), SRC-2 (BD Biosciences), SRC-3 (Affinity Bioreagents), RIP140 (Affinity Bioreagents), REA (Upstate). Total actin was detected using anti – β-actin (CALBIOCHEM®) in all cell lines (one representative cell line shown)as a western blot loading control.

2.4.7 Modulation of coactivators and corepressors in MDA-MB-231 cells

(i) Effect of E_2 on cofactor mRNA expression in MDA-MB-231 cells

MDA-MB-231 cells were used as a comparison of an ERα negative cell line to the receptor expressing MCF-7, LCC-1/2/9 and LY2 cells. All studied cofactors were present as detected by mRNA. Most remarkable was the particularly low constitutive expression of SRC-3 (figure 2.36 A And B). This observation is well described (Anzick, S.L. et al. 1997 and Thenot, S. et al. 1999). Nothing atypical could be identified for the expression of other cofactors. Unusually, as observed in LCC-9 and LY2 cells, the presence of oestrogen produced a small down-regulation between 0h and 6h for some cofactors despite the fact that MDA-MB-231 cells do not express ERα though they do express ERβ. Significant reductions were observed in NCoR (1.8 fold) and for p300 (1.3 fold). SMRT expression did not follow this trend.

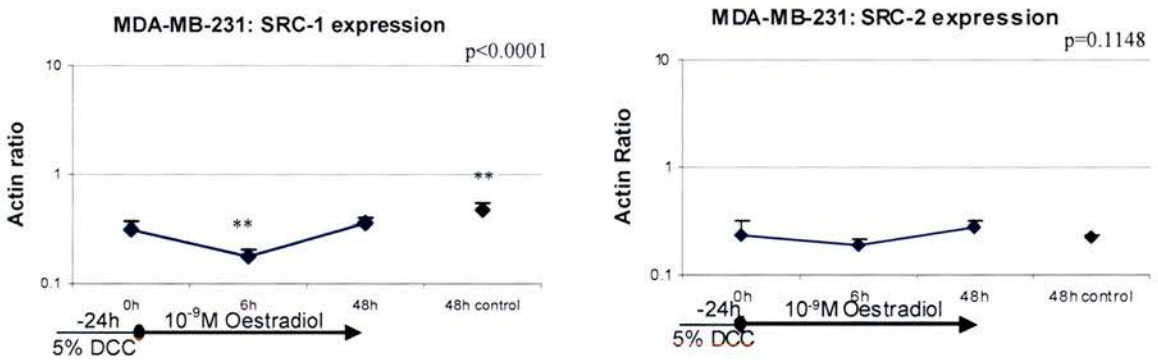


Figure 2.35 A: mRNA expression of p160 in MDA-MB-231 cells. Cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2. RNA was collected at 0h (72h after plating before treatment) 6h and 48h. A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=p<0.05, **=p<0.01.

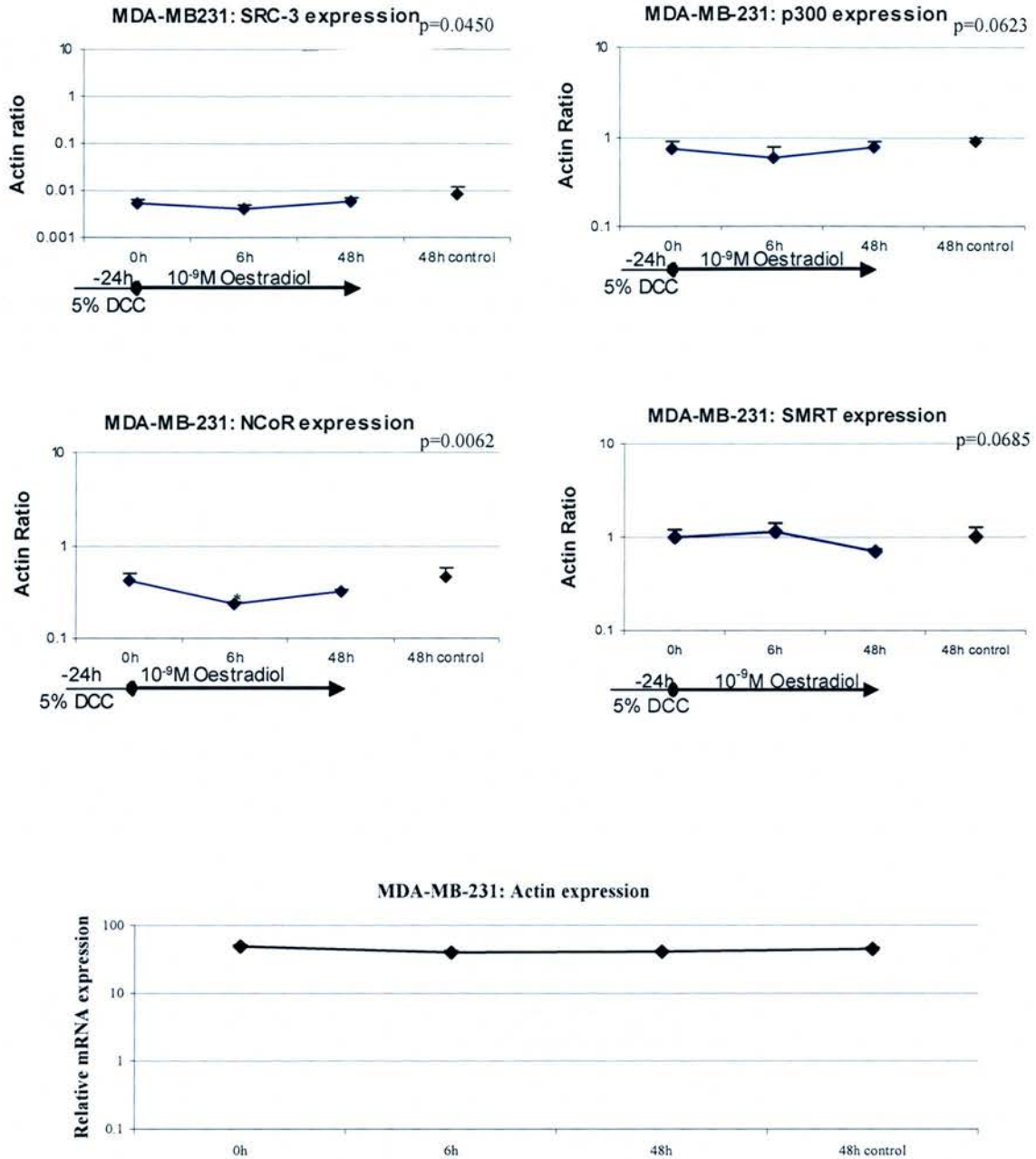


Figure 2.35 B: mRNA expression of cofactors in MDA-MB-231 cells. Cells were seeded in complete media for 24h and placed in reduced media conditions for 48h before treatment with 10^{-9}M E_2. RNA was collected at 0h (72h after plating before treatment) 6h and 48h. A representative experiment is shown of at least two experiments carried out. Each point represents mean of triplicate PCR analysis for each sample. Significant variance between time points and 0h was determined by one-way ANOVA and Dunnets multiple comparison test where *=$p<0.05$, **=$p<0.01$.

(ii) Effect of E_2 and tamoxifen on cofactor protein expression

The low mRNA expression of SRC-3 in MDA-MB-231 cells was reflected in low protein levels. SRC-3 protein was almost undetectable (figure 2.37). In contrast, SRC-2 bands representing the 160 kDa product were detected in a uniform fashion across all treatment groups. The same could be revealed about SRC-1. Unlike observations in LCC-2 cells where the protein product revealed variations within the double bands, a consistently strong upper band and an equally consistent faint lower band was observed. This uniform band expression for multiple band products was also detected in RIP140. Overall, in MDA-MB-231 cells, cofactors protein levels were not modulated at 48h by the addition of E_2 or tamoxifen.

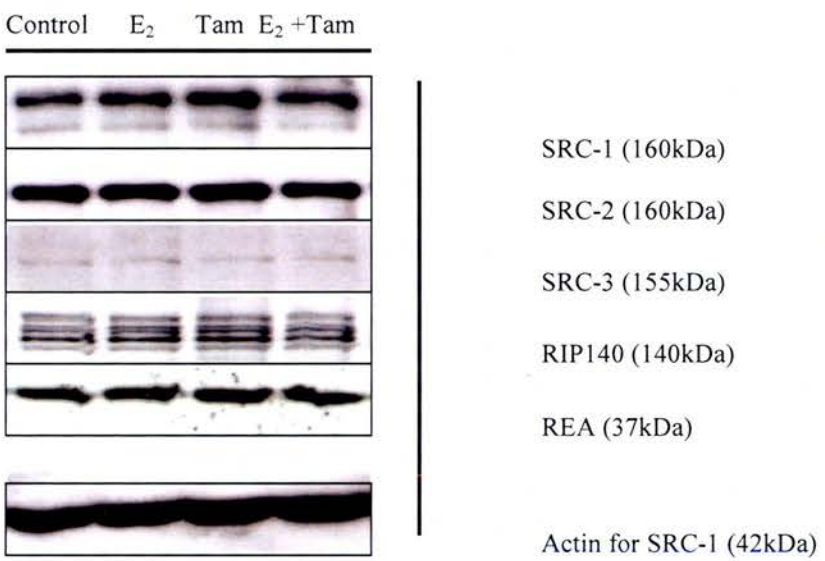


Figure 2.37: Western blot analysis of several cofactors in MDA-MB-231 cells placed in reduced media conditions for 48h before treatment of 10^{-9}M E_2, 10^{-6}M tam or 10^{-9}M E_2 and 10^{-6}M tam. The control group was left untreated. Protein was collected at 48h of treatment. 100μg protein was loaded per lane and detected using anti- SRC-1 (Upstate), SRC-2 (BD Biosciences), SRC-3 (Affinity Bioreagents), RIP140 (Affinity Bioreagents) and REA (Upstate). Total actin was detected using anti – β-actin (CALBIOCHEM®) in all cell lines (one representative cell line shown) as a western blot loading control.

	MCF-7	LCC-1	LCC-9
SRC-1	xxx	xxx	xxx
SRC-2	xx	xx	xx
SRC-3	xxx	xxx	xx
p300	xx	xxx	xx
RIP140	xx	xxxx	xxxx
NCoR	xxx	xxxx	xxx
SMRT	xxx	xxx	xx

Table 2.5 A: Summary of coregulator baseline mRNA expression levels in MCF-7, LCC-1 and LCC-9 cells. Actin ratio is shown where x: <0.1; xx: 0.1-1; xxx: 1-2; xxxx: >2.

	MCF-7			LCC-1			LCC-9		
	E_2	T	ET	E_2	T	ET	E_2	T	ET
SRC-1	→	→	→	→	→	→	→	→	→
SRC-2	↓ / →	↓ / →	↓ / →	→	→	→	→	→	→
SRC-3	→	→	→	→	→	→	→	→	→
p300	→	→	→	→ / ↑	→	→	→	→	→
RIP140	↑	→	→	→ / ↑	→ / ↑	→ / ↑	→	→	→
NCoR	↑	→ / ↑	→/ ↑	→	→	→	→	↓ / →	↓ / →
SMRT	→	→	→	→	→	→	→	→	→

Table 2.5 B: Changes of cofactor mRNA expression in response to E_2, tamoxifen (T) or E_2 plus tamoxifen (ET); control (C): no change (→), significant decrease (↓), significant increase (↑). Two symbols separated by '/' are only depicted where 6h results vary from 48h results.

2.4.6 Discussion

The function of ERα is modulated by interaction with a growing number of coregulatory proteins. Coactivators enhance gene transcription, corepressors inhibit gene transcription. Two regions, AF-1 and AF-2 within the A/B and E domain of the ERα, respectively, have been identified as docking sites for coregulators (Klinge, C.M. 2000). Numerous studies have produced evidence that coregulators interact directly with the ERα and are part of a large multi-component complex (Rosenfeld, M.G. and Glass, C.K. 2001, Glass, C.K and Rosenfeld, M.G. 2000). This allows coregulators to modulate chromatin organisation and recruit basal transcription

factors initiating or repressing transcription and has led to speculation as to whether changes in cofactors expression and functionality could be involved in the development of breast cancer and/or the development of antioestrogen resistance.

The p160 family of coactivators is a group of three homologous coactivators which serve as an ideal case to study potential distinct functionality and involvement in malignant development. Involvement in breast cancer has primarily been demonstrated for SRC-3. SRC-3 gene expression has been reported in normal and malignant breast tissue and amplification in breast cancer relative to normal tissue has been detected of varying magnitude (reviewed in Klinge, C.M. 2000). Amplified SRC-3 expression has been correlated with tumour size and grade (Bouras, T. *et al.* 2001) as well as ER positivity *in vitro* and *in vivo* (Bautista, S. *et al.* 1998; Azorsa, D.O. *et al.* 2001), although other researchers suggest an inverse correlation between SRC-3 and ER amplification (Bouras, T. *et al.* 2001). In MCF-7 cells, SRC-3 mRNA and protein are highly expressed (List, H.J. *et al.* 2001 and Thenot, S. *et al.* 1999). Expression has been shown to be unaffected by E_2 or tamoxifen in these cells (Cremoux, P. *et al.* 2003). The same study analysed expression in LCC-1, LCC-2 and LCC-9 cells and found comparable levels of SRC-3 mRNA. Low levels of SRC-3 mRNA have been reported for ER-negative MDA-MB-231 cells and protein expression of p160 proteins was determined to be weaker in MDA-MB-231 than in MCF-7 cells (Thenot, S. *et al.* 1999).

mRNA transcription of all SRC family members in MCF-7 cells was confirmed in this study. It was established that the resistant cell lines LCC-1, LCC-2 and LCC-9 and LY2 express comparable levels of SRC-1 and SRC-2 (see comparison table 2.5 A). SRC-3 was considerably lower in the resistant lines as well as in MDA-MB-231 cells without E_2 and tamoxifen. Interestingly, E_2 caused a short-lasting down-regulation of mRNA expression after 3h and up to 6h in LCC-2, LCC-9 and to some extent in LY-2 cells in most cases of the three SRC mRNA. This contrasts with expression in MCF-7 cells where E_2 had no effect and LCC-1 cells where E_2 exposure led to a small increase after 1h with a return to baseline after 6h. Baseline protein expression differences for all three SRC members were detected between parental and resistant cell lines. While SRC-1 protein was absent or was detected only weakly in MCF-7, LCC-9 and LY2 cells, it appeared more strongly in

LCC-2 and in LCC-1 cells. SRC-2 expression was particularly high in LCC-2, LCC-9 and LY-2 cells while SRC-3 was expressed consistently in all cell lines except MDA-MB-231 where expression was low. In general, neither E_2 nor tamoxifen seem to impact on SRC protein expression with notable exceptions. For example, SRC-1 was strongly induced by both ligands in LCC-1 cells. E_2 acted as a weak antagonist for both SRC-2 and SRC-3 mRNA expression and SRC-2 protein expression in LCC-2 cells.

While there is no doubt that p160 proteins are involved in ER transcription, it remains unclear whether transcription is dependent upon the presence and functionality of individual members or whether members are able to compensate for one another (Xu, J. and Li, Q. 2003 (b)). Distinct functionality has primarily been shown within *in vitro* experiments. For example, in T47D reporter transfection studies, progesterone and glucocorticoid receptors showed selective recruitment of SRC-1 and SRC-2, respectively, leading to differential recruitment of downstream coregulatory factors and differential histone modification (Li, X. *et al.* 2003). A detailed study analysing direct target genes of SRC-3 identified 18 strong and 11 weak distinct genomic binding sites all involving the binding of ERα (Labhart, P. *et al.* 2005). SRC-3 responsive sites were located upstream, within or even downstream of the target genes. ERα-SRC-3 binding was promoted by oestrogen on several but not all sites. This is evidence for a target gene, receptor and ligand specific role of coactivator SRC-3. *In vivo*, SRC-3 has shown tissue specific expression and suppression of the cofactor has resulted in disrupted aspects of female reproductive development including delayed puberty and attenuated mammary development in mice (Xu, J. *et al.* 2000). Loss of SRC-3 expression has been associated with reduced tumour incidence and delayed latency in oncogene *v*-Ha-*ras* expressing transgenic mice independent of ovarian hormones (Kuang, S.-Q. *et al.* 2004). On the other hand, SRC knockout mice experiments have demonstrated that SRC members do exhibit specific functions but are nevertheless able to compensate for each other. Structural homology between SRC family members would support partial compensatory behaviour. SRC-1 deficient mice are viable and fertile although they exhibit decreased growth in several reproductive organs (Xu, J. *et al.* 1998). Interestingly, SRC-1 null mutants express elevated levels of SRC-2 in several regions of the brain

and other tissues compared to normal animals. Another study supports this finding. In the rat brain, oestrogen dependent progesterone receptor synthesis is inhibited by antisense to SRC-1 and SCR-2 resulting in blocked reproductive behaviour (Apostolakis, E.M. *et al.* 2002). Normal reproductive behaviour was displayed by SRC-1 knock-out mice suggesting that up-regulated SRC-2 might compensate for the SRC-1 deficiency. Cofactor expression profiles and oestrogen and antioestrogen modulation has been established to be specific for each of the resistant and sensitive phenotypes. This could demonstrate distinct roles for SRC coactivators in these tissues. At the same time, altered oestrogen signalling might eliminate the expression and/or function of one SRC coactivator while another SRC member with its particular functions may take its place. Over expression of this cofactor would be apparent. Antisense experiments selectively inhibiting individual members would help to confirm the latter hypothesis.

Altered SRC mRNA expression does not always correlate with changes in protein expression at 48h. This observation can in general also be found in the literature. High SRC-3 mRNA expression does not necessarily translate into elevated protein levels in breast cancer tissue (List, H.J. *et al.* 2001). This indicates that ER signalling changes that manifest in resistant phenotypes may not influence mRNA expression but could determine SRC cofactor levels in a post-translational manner. Differential SRC involvement in ER regulated transcription might take place through altered protein production, turnover and breakdown. Protein expression studies are also not sufficient to detect changes in protein activity. It is known that oestrogen receptor activation can be accomplished by complex signalling between growth factor pathways. For example, activation of one of the ErbB receptors by EGF can in turn activate ERα via SER^{118} phosphorylation by MAPK (through ERK1/2) is just one example that has been described (Kato, S. *et al.* 1995, reviewed by Driggers, P.H. and Segars, J.H. 2002). However, it is not just the ERα itself that is prone to direct cross-activation by other signalling pathways such as the ERK1/2 mitogen-activated protein kinase pathway (MAPK). It has been demonstrated that the AD2 region within SRC-3 is responsive and can be directly activated by MAPK/ERK phosphorylation in MCF-7 cells (Font de Mora J. and Brown, M. 2000). This

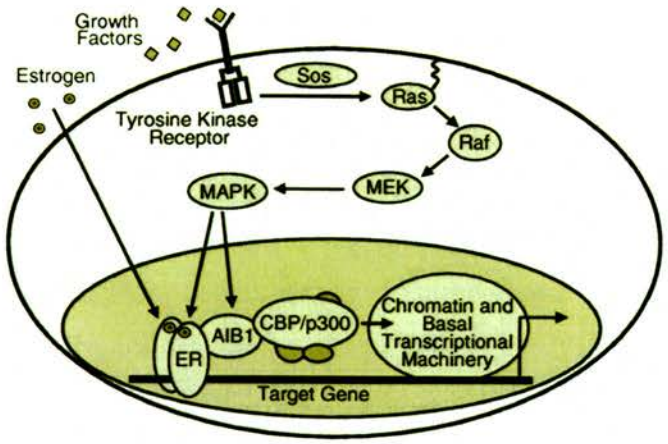


Figure 2.38: Alternate activation of SRC-3/AIB1 by MAPK phosphorylation to modulate gene transcription (Font de Mora, J. and Brown, M. 2000).

implicates a much more complex cross talk where cofactors such as SRC-3 can be activated not directly by E_2 but by other growth factor stimulated pathways which in turn then modulate gene transcription via feedback to the ERα (see figure 2.38). To produce further evidence for this mechanism, it was shown that MAPK/ERK phosphorylated SRC-3 demonstrates increased recruitment of p300/CBP, another coactivator able to modulate H3/H4 acetyltransferase activity. This study also failed to detect significant levels of SRC-3 phosphorylation in SRC-3 expressing BT-474 cells, strengthening the argument that cofactor expression levels alone do not necessarily correspond to heightened cofactor activity and relative levels are not an indicator of active involvement in gene transcription.

If SRC-3 is activated by the MAPK pathway in a manner similar to ER activation to stimulate gene transcription, it could also be actively involved in antioestrogen resistance. This possibility seems even more plausible since SRC-3 amplification in breast cancer has been linked to overexpression of HER2/neu (or ErbB2), a member of epidermal growth factor (EGF) receptor family able to activate the MAPK/ERK signalling cascade (Bouras, T. *et al.* 2001). An earlier study suggested that HER2/neu overexpression correlates with a low level of tamoxifen resistance in MCF-7 sublines (Benz, C.C. *et al.* 1993). This HER2/neu overexpressing cells subline, MCF-7/HER2-18, demonstrates rapid tumour growth insensitive to tamoxifen in overiectomized athymic nude mice in the presence of oestrogen. In contrast, in a separate study by different investigators, MCF-7 variant

cells resistant to antioestrogens tamoxifen, ICI 164,384 and ICI 182,780, failed to express altered levels of ErbB-family receptors including HER2/neu, and oestrogen as well as antioestrogens regulated HER2/neu in a similar pattern than the parental cells (Larsen, S.S. *et al.* 1999). Further, inhibition of Her-2/neu receptor signalling with Herceptin, an antibody to this receptor, did not modulate cell growth indicating that activation of the HER2/neu signalling pathway alone does not lead to antieostrogen resistance. Interestingly, in transfected HER2/neu overexpressing MCF-7 cells, activation of the HER2/neu pathway by addition of the ligand heregulin 1-beta1 diminished growth inhibitory effects of ICI182,780 demonstrating that both, overexpression of HER2/neu and ligand actived receptor signalling are necessary to alter antioestrogenic effects of ICI182,780. In tamoxifen resistant -also HER2/neu overexpressing MCF-7 cells, it has been shown that after inhibition of HER2/neu and MAPK signalling, tamoxifen can regain antagonistic characteristics and override resistance (Kurokawa, H. *et al.* 2000).

A recent clinical study assessing SRC-3 protein expression with relation to tumour characteristics and patient outcome concluded that only tumours with high expression of SRC-3 in combination with high expression of HER2/neu show a relative resistance to tamoxifen (Osborne, C.K. *et al.* 2003). High HER2/neu expression alone did not result in reduced tamoxifen treatment benefits compared with low Her2/neu expressing tumours. This would indicate, that only where HER2/neu activated MAPK signalling also activates SRC-3 via phosphorylation instigating feedback to the ER pathway might the effectiveness of tamoxifen as an antioestrogen be modified. Furthermore, in experiments comparing parental E_2 and tamoxifen sensitive MCF-7 cells with E_2 sensitive and tamoxifen resistant MCF-7/HER2-18 neu cells, increased crosstalk between the oestrogen and HER2/neu receptor has been proposed as a mechanism for acquired tamoxifen resistance (Shou, J. *et al.* 2004). Oestrogen and tamoxifen act as agonists in the HER2/neu overexpressing cells stimulating tumour growth in xenograft models. Enhancing signalling between EGFR/HER2 and E_2 receptor pathways is accomplished by cross-phosphorylation of both receptors but also MAPK/ERK mediators ERK1/2 and Akt as well as SRC-3. Tamoxifen and E_2 bound ER recruit SRC-3 in resistant MCF-7/HER2-18 cells whereas in parental MCF-7 cells, tamoxifen recruits corepressors

NCoR and HDAC giving SRC-3 a pivotal role in the development of tamoxifen resistance.

Our understanding of the intricate oestrogen and tyrosine kinase receptor signalling network is growing as research progresses. Communication takes place via an increasing number of other pathways including ligands EGF or heregulin, signal transducers Ras and Raf and the ERK1 and ERK2 activated MAPK pathway in the case of the ErbB network (Driggers, P.H. and Segars, J.H. 2002). Traditional activation of the ER by its ligand E_2 has long been shown to be only one way of transcriptional regulation. As functional consequences of this crosstalk are being discovered, new strategies will become available to combat this acquired hormone and antihormone resistance. In addition, it becomes apparent that monotherapy targeting single growth factor pathways is unlikely to be optimal treatment (Shou, J. *et al.* 2004).

The discovery of corepressors, transcriptional coregulators repressing oestrogen receptor mediated transcription, led to the obvious suggestion that the functional absence of these coregulators might allow amplified transcriptional enhancement by coactivators thereby promoting breast cancer cell growth. Corepressors such as NCoR and SMRT are generally thought to impose their regulatory function by association with the ER and recruiting other coregulators and transcription factors to form a repressive transcription complex but have also been shown to act through other mechanisms such as actively blocking ER helix 12, a coactivator binding site, to prevent transcriptional activation (reviewed in Dobrzycka, K.M. *et al.* 2003). Tamoxifen mediated ER transcription has therefore been suggested to involve the predominant recruitment of corepressors and the expression of such coregulators might be predictive of tamoxifen response in breast cancer. Corepressor association with the ER has been firmly established in the presence of an antagonist. NCoR and SMRT have been shown to interact with helices H3/H4 within the LBD of ERα in the presence of *trans*-hydrotamoxifen (TOT), a tamoxifen metabolite (Yamamoto, Y. *et al.* 2001). Reduced corepressor interaction was observed in a helix H3 ERα mutant where TOT acts as an agonist. NCoR is reported to immunoprecipitate strongly only in the presence of TOT in parental MCF-7 cells (Lavinsky, R.M. *et al.* 1998). ERα has also been shown to

actively recruit NCoR and SMRT into large transcription complexes at E_2 responsive genes cathepsin D and pS2 where tamoxifen is present as an antagonist (Shang, Y. *et al.* 2000). However, unaltered ER transcriptional activity was demonstrated in MCF-7D cells, an ER positive but NCoR negative MCF-7 clone (Morrison, A.J. *et al.* 2003). Based on the before -mentioned findings, treatment with tamoxifen might be expected to increase E_2 responsive gene transcription and cell proliferation in the absence of NCoR. This might imply a shift in coregulators complex assembly and the use of alternative corepressors such as the structurally similar SMRT in such mutant cells to control cell growth.

Most proposed corepressor mediated ER transcription mechanisms are based on *in vitro* studies. *In vivo* investigations are infrequent and provide more conflicting findings. In a mouse model, consistent exposure to tamoxifen not only leads to tamoxifen resistance but significantly reduced NCoR expression suggesting that reduced NCoR expression or blocked NCoR binding enables the antioestrogen to increase cell proliferation (Lavinsky, R.M. *et al.* 1998). Low NCoR mRNA expression also correlated with significantly shorter relapse –free survival in ER positive breast cancer patients suggesting a possible predictive role for NCoR with respect to endocrine treatment benefit (Girault, I. *et al.* 2003). Other *in vivo* studies have so far failed to demonstrate specific tamoxifen resistant corepressor expression patterns for SMRT. A small investigation of 19 tamoxifen resistant breast tumour samples failed to detect mRNA expression differences of corepressor levels including SMRT compared to 21 control tumours (Chan, C.M.W. *et al.* 1999). Short term tamoxifen treated tumours did not reveal different SMRT mRNA levels. The same publication had first mentioned no differences in SMRT mRNA expression *in vitro* for MCF-7 cells and an MCF-7 tamoxifen resistant variant line. If relative expression levels of corepressors are responsible for tamoxifen resistance, lower levels of SMRT might have been present in the resistant tissue samples.

In agreement with Chan, C.M.W. and colleagues (1999), SMRT as well as NCoR mRNA was detected in similar levels in parental MCF-7 and all MCF-7 variant cells irrespective of their oestrogen or antioestrogen sensitivities (see comparison table 2.5 A). Both coregulators were also observed in ER negative MDA-MB-231 cells. Protein expression using Western blotting for both corepressors

was attempted but unsuccessful. Protein expression in these cell lines was too low for accurate analysis. As described for p160 coactivators, a small short-term oestrogen mediated increase in LCC-1 cells and decrease in NCoR mRNA level was noticed in LCC-2, LCC-9 and LY2 cells. Interestingly, tamoxifen had generally no or small effects on either SMRT or NCoR expression in these cell lines (see comparison table 2.5 B). These results oppose the theory that acquired tamoxifen resistance is due a lack of corepressor expression. If these corepressors played a role in tamoxifen resistant growth, lower levels of NCoR and/or SMRT would have been expected in resistant models and tamoxifen treatment in antieostrogen sensitive MCF-7 cells might also have resulted in lower corepressor expression. However, this does not rule out corepressors as part of tamoxifen acquisition. Firstly, NCoR and SMRT are two of a constantly increasing number of identified corepressors. If the function of coregulators determines the cells response to an antioestrogen, it might not be SMRT or NCoR that are important in these cell lines. And secondly, both corepressors were discovered only ten years ago (Chen, J.D. and Evans, R.M. 1995; Horlein, A.J. *et al.* 1995). Since then ongoing research has provided a large amount of information about their role in ER mediated gene transcription but knowledge is still limited. Functional studies analysing corepressor activation might help to elucidate the role of NCoR and SMRT in antioestrogen mediated signalling in MCF-7 cells and the LCC models. For example, similar to findings within the SRC coactivator family, binding was shown to be involved in cross-signalling between ER and ErbB receptors in tamoxifen resistant HER2/neu overexpressing MCF-7 cells (Kurokawa, H. *et al.* 2000). In this study, levels of NCoR were unchanged in the MCF-7 variants but increased NCoR binding was observed when both, HER2/neu and MAPK signalling were inhibited suggesting perhaps not corepressor availability but altered binding capacity determines corepressor participation in tamoxifen resistance.

The coregulator RIP140 illustrates the additional dimension each and every one of the coregulators can add to nuclear receptor function. RIP140 had been identified as a coactivator based on its ability to interact with the LBD of nuclear receptors and specifically with the ER in an oestrogen dependent manner (Cavailles, V. *et al.* 1995). Inconsistent evidence was published in several *in vitro* models questioning whether interaction with the ER did or did not occur in the presence of

antieostrogens such as 4-OHT or ICI182,780 and ICI164,384 (reviewed in Klinge, C.M. 2000). It soon became evident that RIP140 can also modulate transcriptional repression. The direction of transcriptional modulation might be based on several findings. Reporter gene activation by RIP140 was observed in the presence of low concentrations of E_2 but gene repression in the presence of high concentrations of E_2 (Cavailles, V. *et al.* 1995). In addition to the N-terminal coactivator common LXXLL motif, a further ER interacting site within the C-terminal of RIP140 was identified (L'Horset, F. *et al.* 1996). Different ERα binding sites were shown to associate with the coregulator and receptor specific binding affinities were demonstrated (Peters, G.A. and Khan, S.A.1999; Kumar, M.B. *et al.* 1999). Most significantly, a study indicated that the function of RIP140 is sensitive to histone deacetylase inhibitors (Wei, L.N. *et al.* 2000). It is reported that RIP140 is able to directly recruit HDAC1 and HDAC3 to its N-terminal interacting site leaving no doubt that this protein can modulate in a bidirectional manner either repressing or activating gene transcription. Coregulators such as RIP140 are often called negative coregulators or corepressors because they directly antagonize coactivators and compete for association with AF-2, the traditional coactivator binding site (Nilsson, S. *et al.* 2001).

Based on these diverse functions of RIP140, it is perhaps not surprising that evidence for this protein as a marker of tamoxifen resistance is rare and conflicting. It appears that the function of this coregulator is particularly tissue and promoter specific and dependent on the availability of ligands or other coregulatory proteins. Several studies state subtle or no variations of RIP140 mRNA and protein expression between cells of different diseases as well as in-between different breast cancer tissues. The coregulator has been reported to be expressed in HeLa, ZR75-1 and MCF-7 breast cancer cells (Cavailles, V. *et al.* 1994). A comparison between breast cancer and endometrial cancer cells revealed uniform expression of RIP140 mRNA and protein across the panel of seven cell lines including the ERα negative MDA-MB-231 cells (Thenot, S. *et al.* 1999). In this study, expression of RIP140 is stimulated 2-3 fold by oestrogen and reduced by 4-OHT and ICI164,384 in MCF-7 cells. This modulation is reported to be rapid and persisting over a period of 24h and is suggested to be independent of de novo protein synthesis. However, a separate

study comparing parental MCF-7 cells and a tamoxifen resistant variant does not observe this oestrogen stimulation in the parent line (Chan, C.M.W. *et al.* 1999). Basal levels of RIP140 mRNA are lower in the resistant MCF-7 cells and oestrogen stimulation is of small magnitude. This result was confirmed for RIP140 mRNA levels in a panel of untreated and short term tamoxifen treated as well as tamoxifen resistant breast tumours.

In this study, RIP140 mRNA expression was generally slightly higher in tamoxifen resistant LCC-1, LCC-2 and LCC-9 cells compared to the MCF-7 parent line (see comparison table 2.5 A). This contrasts with previously mentioned publications where either no differences were reported or lower expression was associated with tamoxifen resistance. It needs to be considered that in this experiment RIP140 mRNA expression was compared between MCF-7 and several tamoxifen resistant lines. MCF-7 cells only represent one tamoxifen sensitive breast cancer phenotype and conclusions with respect to coregulators as a marker of antioestrogen resistance without analysis of other antioestrogen models would be premature. Oestrogen clearly stimulated the expression of RIP140 in MCF-7 cells short term at 6h as well as long term at 48h but remained unaffected by the antioestrogen tamoxifen. The fact that some laboratories do not find any or only small oestrogen stimulation might partially be due to the initially small induction. The magnitude of the response did not become apparent until 48h of treatment was applied. Some oestrogen stimulation was observed in all MCF-7 variant lines including LY2 cells but not LCC-9 cells (see comparison table 2.5 B). Unlike MCF-7 cells, this stimulation was found to be a late effect and was not observed until 48h. A delayed increase in RIP140 mRNA was also observed when tamoxifen was added. RIP140 protein was detectable in similar levels in all cell lines. This might imply that changes in expression at the translational level are generally not responsible for the acquisition of tamoxifen resistance. Neither oestrogen nor tamoxifen had an effect on expression with the exception of LCC-2 cells where oestrogen as well as oestrogen in combination with tamoxifen increased protein expression. Results corresponded to observations at the mRNA level. Detection of varying multiple bands for RIP140 could suggest an additional element of RIP140 where the expression of different isoforms and splice variants might determine the functionality of the protein in a

particular tissue type and its response to endocrine agents. It could also be the results of posttranslational modifications such as phosphorylation.

These results suggest that expression of individual coactivators or corepressors determine the phenotype of breast cancer tissue with respect to endocrine response. In most cases variations in basal expression are subtle and response to oestrogen or tamoxifen cannot always be linked to the tissues proliferative behaviour. There is no doubt that each one of the coregulators plays a significant role in normal and malignant development. Rather then the absence or misfunction of corepressors in the case of malignant development, it has been suggested that a balance of coactivators and corepressors might define the response to oestrogen and antioestrogens. In this case, analysis of individual cofactors might provide limited information. The specificity of transcriptional regulation for each cofactor is dependent the promoter-receptor complex in its cellular environment. The availability of large numbers of coregulatory proteins and the complex receptor cross-talk makes the mechanism of oestrogen dependent gene transcription extremely versatile.

2.5 Identification and recruitment of oestrogen regulated transcription complexes at the pS2 promoter

2.5.1 Introduction

Chromatin immunoprecipitation (ChIP) was used to investigate the activation process at the pS2 promoter. The pS2 gene had previously been identified to reflect the oestrogen-regulated growth pattern observed in the MCF-7 wild-type and variant breast cancer cell lines. This experiment examined gene activation as indicated by H4 acetylation and sought to identify ERα binding as well as cofactor SRC-1 and SRC-3 recruitment in response to oestrogen. Based on recent reports suggesting a dynamic recruitment assembly at oestrogen responsive promoters such as pS2 (Shang, Y. *et al.* 2000; Métivier, R. *et al.* 2003), a 90 min time course was carried out to gain insight into the recruitment kinetics of transcription components in the resistant cell lines. The experiment used the oestrogen sensitive MCF-7 and the related oestrogen independent LCC-1 and LCC-9 cell lines.

MCF-7 cells were seeded in complete medium, washed in PBS and incubated in reduced media for 48h to ensure an oestrogen-deprived environment. LCC-1 and LCC-9 cells were plated directly into reduced media and incubated for 24h. Cells were treated with 10^{-7}M E_2 for a maximum of 90min. DNA-binding proteins were cross-linked to the DNA with the addition of formaldehyde every 10min and the DNA was sonicated into fragments of 500-1000bp. Fragments were examined using specific antibodies to immunoprecipitate acetylated histone H4, ERα and cofactors SRC-1 and SRC-3. Heat was applied to reverse crosslinks and DNA purified from the samples. Quantitative PCR with pS2 specific primers was used to analyze pS2 promoter fragments with binding of the proteins of interest. A graphic representation of the technique is shown in figure 2.39. For more details of the methodology see section 4.2.4.

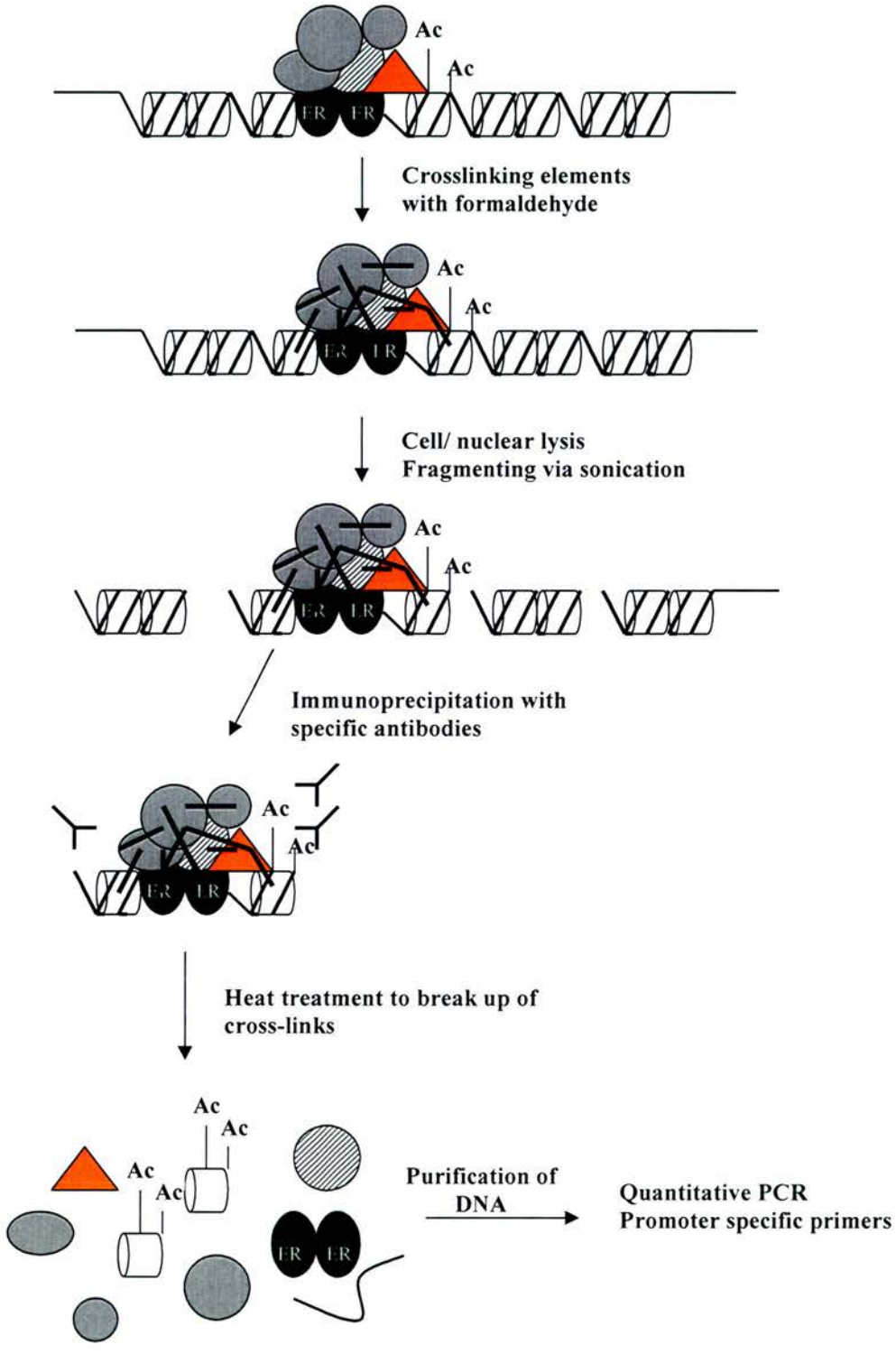


Figure 2.39: Schematic diagram of ChIP technique. Ac = acetylated histones. See text for details.

2.5.2 Optimization of ChIP assay

(i) Optimisation of DNA fragmentation

The protocol for this assay is largely based on guidelines published by the cell signalling company Upstate®. Optimization for use with MCF-7 and MCF-7 variant cell lines was necessary. DNA fragmentation resulting in the majority of sequences containing 500-1000 base pairs was essential. Longer fragments would have increased the background and decreased the solubilization of the chromatin from ruptured cells. Furthermore, it would have made it difficult to distinguish a DNA sequence where a particular protein is bound from a nearby promoter sequence (Aparicio, O. *et al.*, 2004). Shearing of DNA was carried out using different sonication intensities as well as pulse length for cells of all cell lines. After sonication and centrifugation, the DNA was precipitated during phenol-chloroform extraction and the size of DNA fragments was estimated on an agarose gel (for more details see *Materials and Methods* (4.2.4 b.)). The desired fragment length could be achieved with 3 sets of 10 second pulses and the sonicator set to 4Aμ as documented for MCF-7 cells in figure 2.40.

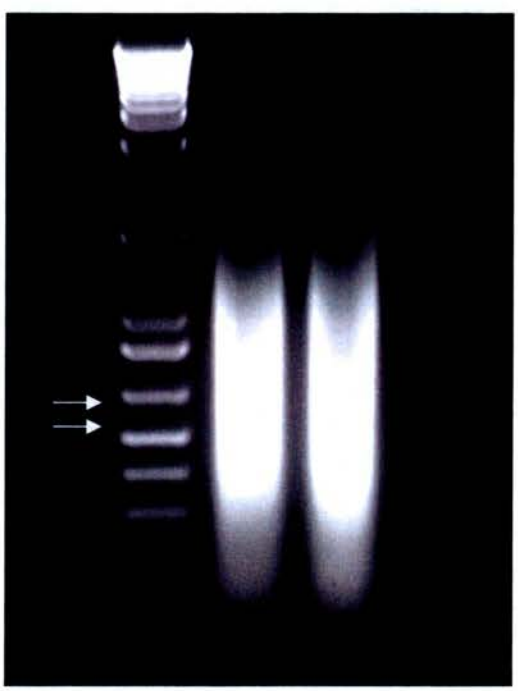


Figure 2.40 : Optimization of DNA fragmentation. Two separate MCF-7 samples, treated with 10^{-9} M E_2 for 40min, were sonicated 3x10sec at 4μA and phenol-chloroform extracted. Similar experiments were carried out for other cell lines used in this project.

(ii) Selection of antibodies for immunoprecipitation

To determine the binding efficiency and concentration of antibodies to be used for immunoprecipitations was also of fundamental importance. In cross-linked chromatin, antibody binding may be less efficient because epitopes are less accessible as binding sites (Orlando, V. 2000). In addition, chromatin immunoprecipitation is used to precipitate several transcription factors associated with the chromatin packed promoter. Association of such bound proteins to the DNA can mask epitopes and produce misleading results where antibodies couple with freely available proteins while bound proteins remain obscured (Aparicio, O. *et al.*, 2004). It was therefore essential to identify antibodies that could be used for binding to components in the chromatin bound complex. For that reason polyclonal antibodies were the preferred option as such antibodies often recognize multiple determinants within a protein.

For detection of histone modifications it was important to use antibodies able to bind to a range of residues as opposed to a specific single residue binding to increase the probability of identifying the modification. The antibody was required to identify acetylated isoforms as opposed to methylated or phosphorylated forms. Modification by acetylation exposes the DNA to restriction enzymes and transcriptional cofactors and is therefore an indicator of transcriptional activation (reviewed in Li, Y.-J. *et al.* 2004). Several antibodies were tested in MCF-7 cells. Anti-acetylated H4 06-866 was chosen for its binding efficiency and ability to detect a span of 19 aa, between the amino acids 2-19 (figure 2.41 and 2.42). Similar experiments were carried out for cofactor antibodies. Optimization for SRC-3 (AIB-1/RAC3) as an example is shown in figure 2.43. The most efficient binding antibody identified for anti- SRC-3 was the polyclonal anti-SRC-3 antibody designated RAC-3 C-20 (figure 2.43 (A)). In addition, concentration curves were constructed to ensure an excess of antibody in each reaction for optimal binding and precipitation efficiency. An example is given in Figure 2.43 (B) where the optimal concentration for each reaction with the SRC-3 antibody was established to be 1μg/ml.

Two control samples are included during each immunoprecipitation. The first is a 'no-antibody' sample that serves as a negative control ensuring specific antibody

Histone Modification H4

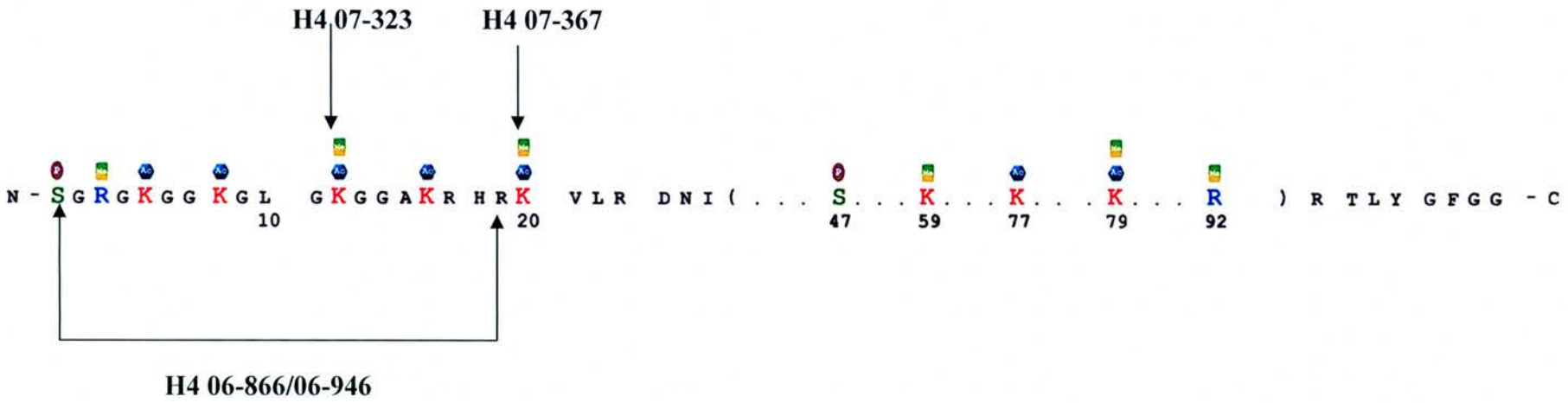


Figure 2.41: Graphical representation of histone modifications (P=phosphorylation , Ac=acetylation, Me= methylation). Binding sites of several Anti-H4 antibodies used in the experiment are indicated above and below (figure taken from www.histone. com published by Upstate®).

interaction with the protein of interest. The second sample is a parallel immunoprecipitation with lysates of the parental MCF-7 cell line. The anti-acetylated H4 antibody was used to ensure successful immunoprecipitation when examining other cell lines and using other antibodies. This is particularly important for comparison with precipitation of non-histone proteins where apparent immunoprecipitation efficiency is much lower.

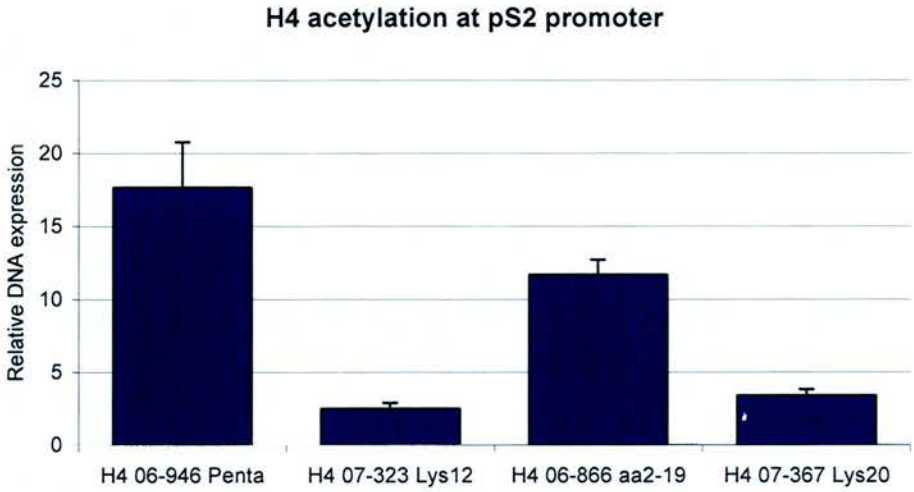


Figure 2.42: ChIP using 45min 10^{-7} M E_2 treated MCF-7 samples and a variety of Histone H4 antibodies at 5µl/ reaction.

(A) (B)

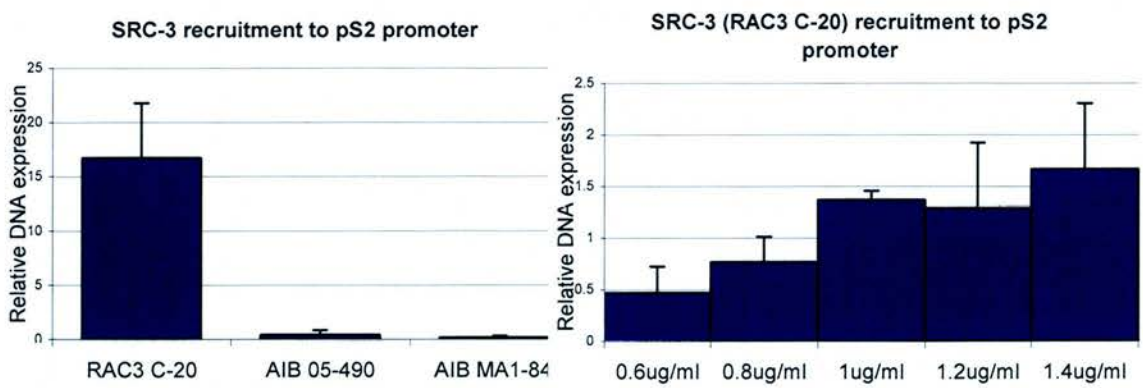


Figure 2.43: **A**. ChIP using MCF-7 samples treated with 10^{-7} M E_2 for 45min immunoprecipitating with various anti-SRC-3 antibodies at 1µg/ml and **B**. ChIP using different SRC-3 (RAC3 C-20) antibody concentrations.

(ii) Primer design

The pS2 gene is approximately 4.5kb long (reviewed in Ribieras, S. *et al.* 1998). Its promoter stretches over 980 nucleotides and starts 26 bases away from the gene start codon (see figure 2.45 (A)). The TATA box and an ERE element are located at nucleotide positions –26 and –392, respectively, from the gene start. The primers used in this experiment amplify a region of 352bp within this promoter starting 93 nucleotides away from the gene start (primers designed and published by Shang, Y. *et al.* 2000) (see figure 2.45 (B)). A second set of primers was designed amplifying a region of 185bp approximately 3kb (nucleotide positions –3446 to –3946 relative to the gene start) upstream from the gene start to ensure specific recruitment of the complex to the ERE of the promoter of interest not to more random regions. This also verifies that the majority of sequences fragmented by sonication had a length of on average 1000bp. As discussed in 2.5.1 (i), larger DNA fragments decrease the resolution between the region where a protein of interest is bound and a region nearby (Aparicio, O. *et al.* 2004). The expression of this region was constitutive and largely independent of E_2. Some H4 acetylation was detected although the acetylation state of the region was much lower. This is due to the less specific binding properties of the histone antibody and the availability of binding sites on the entire genome (Aparicio, O. *et al.* 2004). Binding of ERα or any coactivators could not be detected at this distant site. A representative example of the analysis of pS2 distant region is shown in figure 2.44.

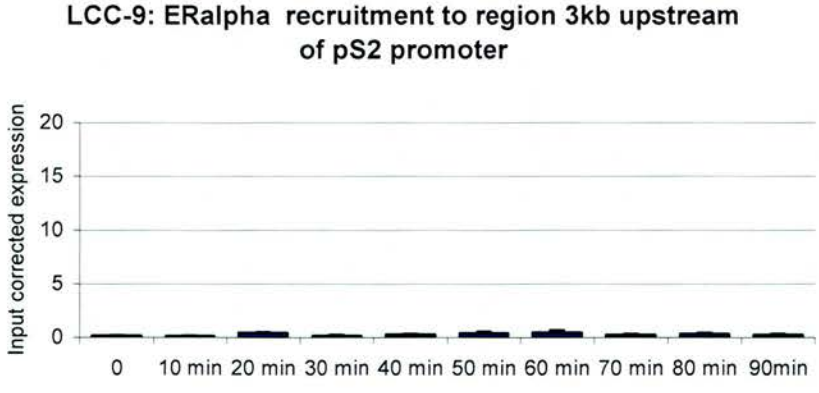
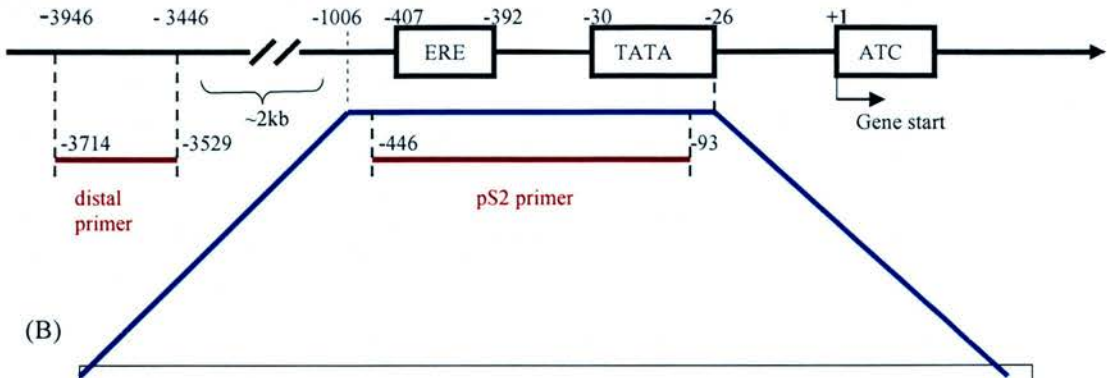


Figure 2.44: ERα recruitment to a region ~3kb upstream form the pS2 promoter. LCC-9 cells were plated in reduced medium (5%DCC in DMEM lacking phenol red) for 24h and treated with 10^{-7}M E_2. Chromatin fraction were collected every ten minutes. Each bar represents the mean of at least triplicate PCR analysis for each sample. Error bars = STD

(A)



(B)

-1006 aaagagaaa

-998 gctccgagca ggggagggga gtgaggcatg gtacaggaga gcaggaggct

-948 gtcctctaaa atacaggagt ccaggggacc aactgggaag gtgtgggagg

-898 gggagggagg gagccccata gacacagggg agtgaaccac gttcactttg

-848 tcagtttttg atggcagctc gtatatacta ttttttttctc cctcctgccc

-898 ccagcccctc ccagaaggag acttaatctg tcgctcaggc tggagtgcag

-748 tagggtgatc tcgactcact gcaacctccg cctcccaggt tcaagtgatt

-698 ctcctgactt aacctccaga gtagctagga ttacaggcac ccgccaccat

-649 gcctggctaa tttttgtatt tttttttttt gtagagacgg ggtttcgcca

-598 tgttggccag gctagtctca aactcctgac tttaagtgat ccgcctgctt

-548 tggcctccca aagtgttggg attacaggcg tgagccactg cgccaggcct

-498 acaatttcat tattaaaaca attccactgt aaaagaatta gcttaggcct

forward primer: **-407**

-448 agacggaatg ggcttcatga gctccttccc ttccccctgc **aaggtcacgg**

-392

-398 **tggcca**cccc gtgagccact gttgtcacgg ccaagccttt ttccggccat

-348 ctctcactat gaatcacttc tgcagtgagt acagtattta ccctggcggg

-298 agggcctctc agatatgagt aggacctgga ttaaggtcag gttggaggag

-248 actcccatgg gaaagaggga ctttctgaat ctcagatccc tcagccaaga

-198 tgacctcacc acatgtcgtc tctgtctatc agcaaatcct tccatgtagc

reverse primer: ***atcacc tctaataaca***

-148 ttgaccatgt ctaggaaaca cctttgataa aaatcagtgg agattattgt

gagtc **-94**

-98 ctcagaggat ccccgggcct ccttaggcaa atgttatcta acgctcttta

-26 **+1**

-48 agcaaacaga gcctgcccta taaaatccgg ggctcgggcg gcctctc a(tc)

Gene start

Figure 2.45: A. pS2 gene organisation and regulatory elements. Promoter and distal primer binding sites are indicated relative to the gene start (+1). B. Nucleotide sequence of the human pS2 promoter. The ERE is shown in **bold.** TATA box is underlined. The binding sites for forward and reverse primers used for ChIP experiments are also underlined (figure source: Lu, D. *et al.* 2001).

(iv) Data analysis

After formaldehyde treatment and sonication but before immunoprecipitation a small fraction of each sample is set aside. These fractions were used as 'input-controls' and serve as comparison for the immunoprecipitated samples. These samples should contain not just DNA fragments selected by antibodies against proteins of interest but the total genomic content. Levels of expression should be unaffected by the addition of E_2 and therefore constant across the sample pool. Formaldehyde crosslinking was reversed and input samples analysed by RT-PCR parallel to experimental samples. Examples of input control RT-PCR results are shown in figure 2.46. Results were then used to normalize experimental samples and account for variations during material collection.

A common standard curve was used for RT-PCR quantification of experimental samples in all ChIP experiments to enable quantitative comparison between cell lines. This curve was constructed using a random MCF-7 input. Employing the same pS2 primers as used in ChIP analysis, the pS2 fragment was amplified, identified and excised from a 1% agarose gel. The isolated pS2 fragments were quantified during a second electrophoretic run with a DNA fragment sizing ladder. The resulting molecular weight of this PCR fragment was used to generate a sample containing $1x10^{10}$ molecules/µl from which a series of dilutions was establish for a standard curve.

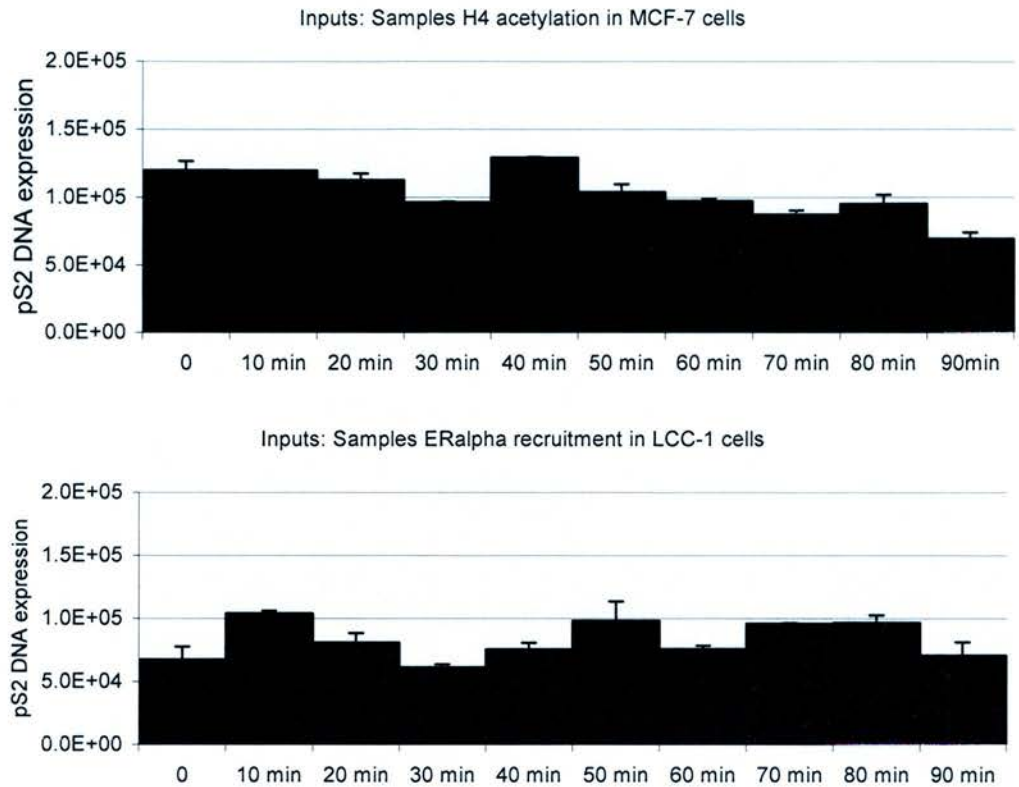


Figure 2.46: pS2 DNA expression of two representative sets of input samples. MCF-7 cells were plated in complete medium (10%FCS in containing phenol red DMEM) for 24h followed by 48h reduced medium (5%DCC in DMEM lacking phenol red) before treatment with 10^{-7}M E_2. LCC-1 cells were plated in reduced medium (5%DCC in DMEM lacking phenol red) for 24h and treated with 10^{-7}M E_2. Chromatin fraction were collected every ten minutes. Each bar represents the mean of at least triplicate PCR analysis for each sample. Error bars = STD.

2.5.3 Oestrogen modulates dynamic cyclic transcription complex assembly in MCF-7 cells

In MCF-7 cells without E_2 treatment (0min), low level H4 acetylation was detected indicating active transcription at the pS2 promoter was detectable (at 0min) (Figure 2.47). H4 acetylation increased upon E_2 treatment after 10min. Two peaks of acetylation were observed, at 30min and 80 min, suggesting two acetylation cycles with a frequency of about 45min within the 90min experiment. Interestingly, during the initial cycle, the level of acetylation was slightly reduced compared to the following cycle and the level of pS2 promoter bound to acetylated H4 appeared to increase in a cumulative manner. Further, during a more detailed time course, the dramatic increase in H4 acetylation in response to E_2 was demonstrated after just 5 min (Figure 2.48). Analysis of samples every 5 min revealed a less efficient subcycle of H4 acetylation reaching a peak at 10 min and, as observed in the 90min time course, subsequently at 30 min.

Active pS2 gene transcription paralleled recruitment of ERα and also coactivators SRC-1 and SRC-3. Addition of E_2 stimulated the recruitment of all three transcriptional components immediately. Within 90min, two cycles of recruitment were detected for ERα, SRC-1 and SRC-3 although peaks did not always coincide with H4 acetylation high points. While ERα recruitment peaks roughly corresponded with H4 acetylation, SRC-1 and SRC-3 promoter occupancy reached a first maximum slightly earlier at about 10min for SRC-1 and at about 20min for SRC-3. The level of recruitment was higher for ERα than the coactivators. Also for ERα, oestrogen produced the greatest fold-increase: immediately after 10min (12.5 fold) and reaching the first peak at 30min (24.2fold). This was due to almost undetectable levels of ERα recruitment in the absence of the ligand.

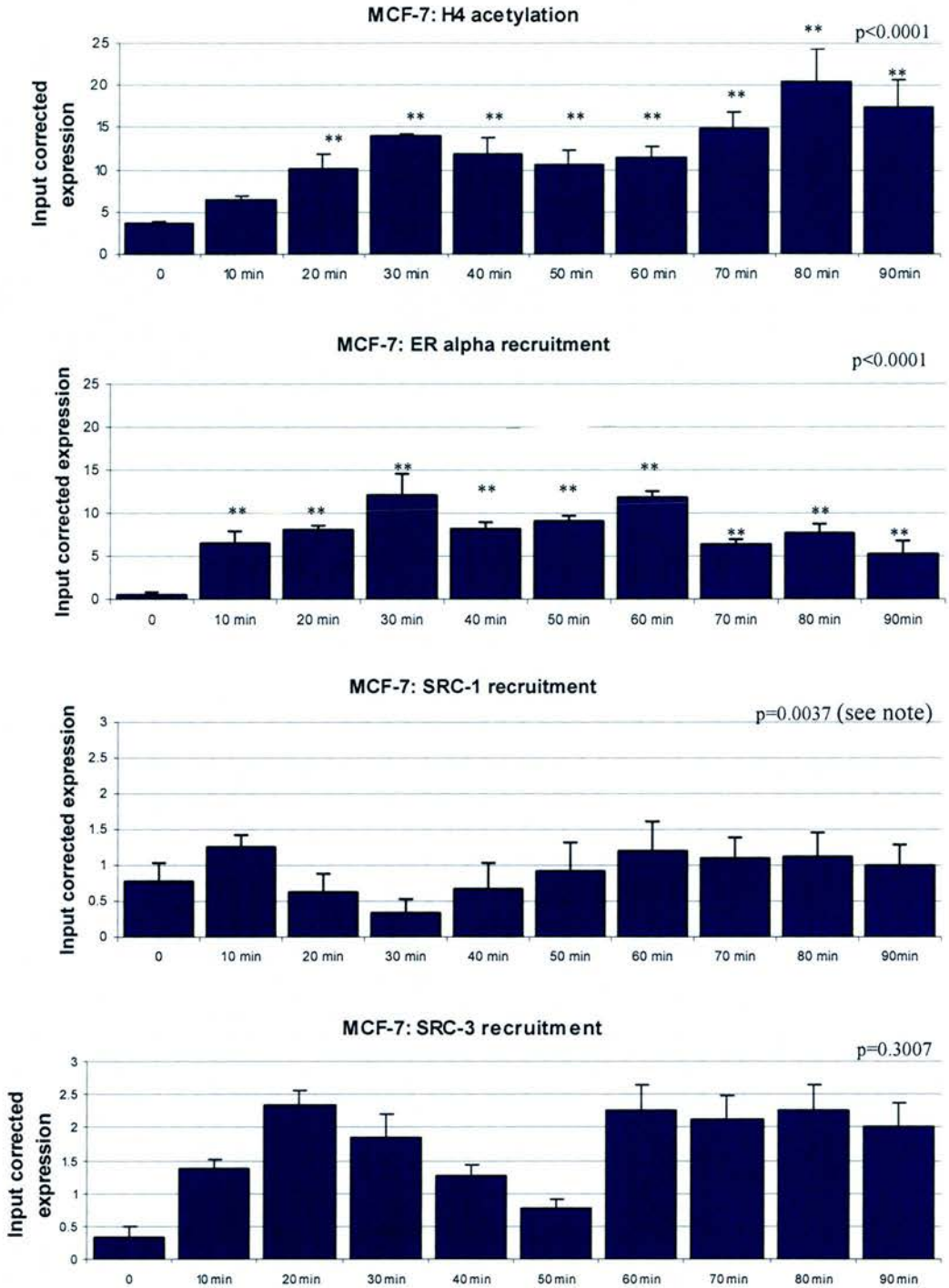


Figure 2.47: H4 acetylation and ERα/SRC-1/SRC-3 recruitment to pS2 promoter in MCF-7 cells. Cells were plated in complete medium (10%FCS in phenol red containing DMEM) for 24h followed by 48h reduced medium (5% DCC in DMEM lacking phenol red) before treatment with 10^{-7}M E_2. Chromatin fraction were prepared every 10min. Data shown are the result of a single collection. Each bar represents the mean of at least triplicate PCR analysis for each sample. Error bars = STD. Significant variance between 0h (no treatment) and time points determined by one-way ANOVA and Dunnets multiple comparison test where *=p<0.05, **=p<0.01. Note: Statistical significance for the recruitment of SRC-1 is only reached comparing data of all time points with 30min, not the control group.

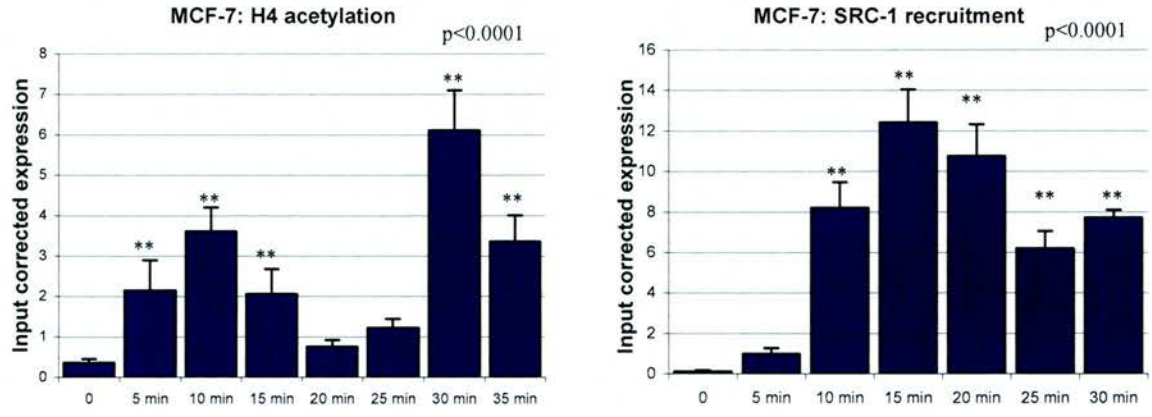


Figure 2.48: H4 acetylation and SRC-1 recruitment to pS2 promoter in MCF-7 cells. Cells were plated in complete serum (10%FCS in phenol red DMEM) for 24h followed by 48h reduced serum (5%ssFBC DMEM lacking phenol red) before treatment with 10^{-7}M E_2. Chromatin fraction were prepared every 5min. Data shown are the result of a single collection. Each bar represents the mean of at least triplicate PCR analysis for each sample. Error bars = STD. Significant variance between 0h (no treatment) and time points determined by one-way ANOVA and Dunnets multiple comparison test where *=p<0.05, **=p<0.01.

2.5.4 Oestrogen differentially modulates dynamic transcription complex assembly in LCC-1 cells

Activation of the pS2 promoter was also revealed to be responsive to oestrogen in LCC-1 cells (Figure 2.49). H4 acetylation reached at least two peaks, at 30min and at 70 min throughout the time course of 90 min. However, cycles of transcriptional activation were not as clearly defined as in MCF-7 cells. Oestrogen mediation on H4 acetylation was less evident reaching first statistical significance at 20min (1.8 fold fold). ERα was recruited to the promoter and association increased immediately after E_2 stimulation (2 fold). Stimulation was weaker than that seen in the parental MCF-7 cell line (compare to12.5 fold, see figure 2.47). ERα recruitment gradually increased to reach a peak at 50 min and decrease up to the 90 min time point.

In addition to ERα, coactivators SRC-1 and SRC-3 associated with the pS2 promoter upon E_2 addition. Compared to MCF-7 cells, higher levels of SRC-1 were found to occupy the promoter. Elevated levels of SRC-1 protein were present at the gene at 0 min and E_2 promoted further recruitment. Promoter occupancy of SRC-3 was increased upon hormone addition similar to the pattern observed in MCF-7 cells. Levels of recruitment were slightly higher than in MCF-7 cells.

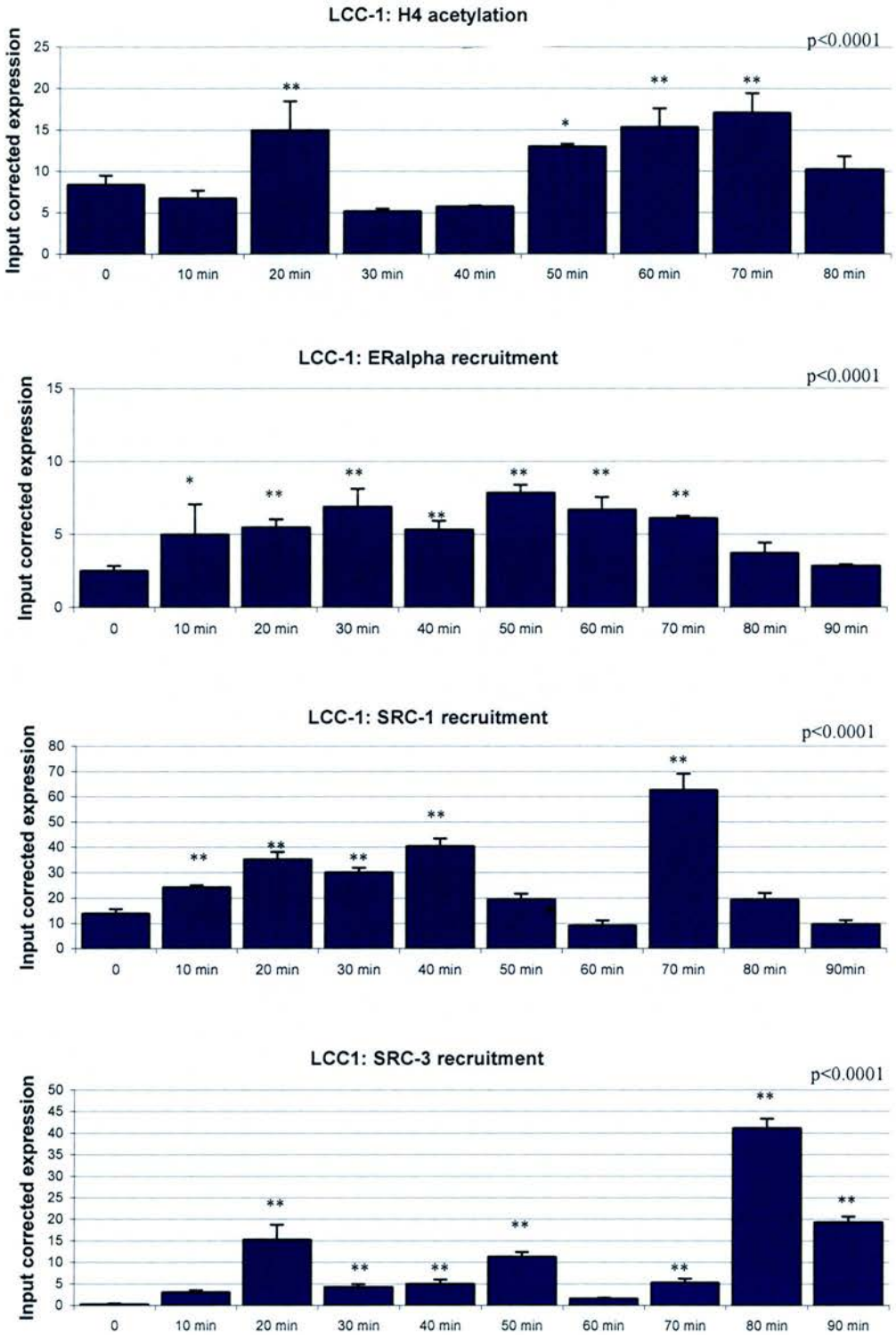


Figure 2.49: H4 acetylation and ERα/ SRC-1 recruitment to pS2 promoter in LCC-1 cells. Cells were plated in reduced serum (5%DCC in DMEM lacking phenol red) for 24h and treated with 10^{-7}M E_2. Chromatin fractions were prepared every ten minutes. Data shown are the result of a single collection. Each bar represents the mean of at least triplicate PCR analysis for each sample. Error bars = STD. Significant variance between 0h (no treatment) and time points determined by one-way ANOVA and Dunnets multiple comparison test where *=$p<0.05$, **=$p<0.01$.

2.5.5 Oestrogen modulates dynamic transcription complex assembly in LCC-9 cells with increased p160 recruitment

In contrast to MCF-7 cells, oestrogen appeared to downregulate H4 acetylation at the pS2 promoter immediately after oestrogen treatment in LCC-9 cells (figure 2.50). High levels of H4 acetylation were detected initially in the absence of the hormone. The first nadir in acetylation was reached at 20min (9 fold) followed by an increase. This acetylation pattern resembles the reverse of gene activation observed in MCF-7 cells where a high point in transcription was reached at about 30 min.

The active promoter was simultaneously occupied by coactivators SRC-1 and SRC-3. Both proteins appeared to be present in the absence of oestrogen. Hormone treatment lead to an immediate dissociation of SRC-1 (3 fold decrease) and SRC-3 (10 fold decrease). After the initial dissociation, a cyclic pattern of recruitment was revealed. Recruitment peaks for SRC-1 as well as SRC-3 roughly coincided with peaks of H4 acetylation. Compared to the parental MCF-7 cells, SRC-1 and SRC-3 recruitment levels were decidedly higher. Unlike the coactivator association, the recruitment pattern of ERα strongly resembled MCF-7 cells where low levels of the receptor were detected in the absence of the ligand and addition of E_2 lead to a marked increase in receptor binding. ERα recruitment peaks appear to coincide with the inactive state of the pS2 promoter as well as with low-level recruitment of SRC-1 and SRC-3.

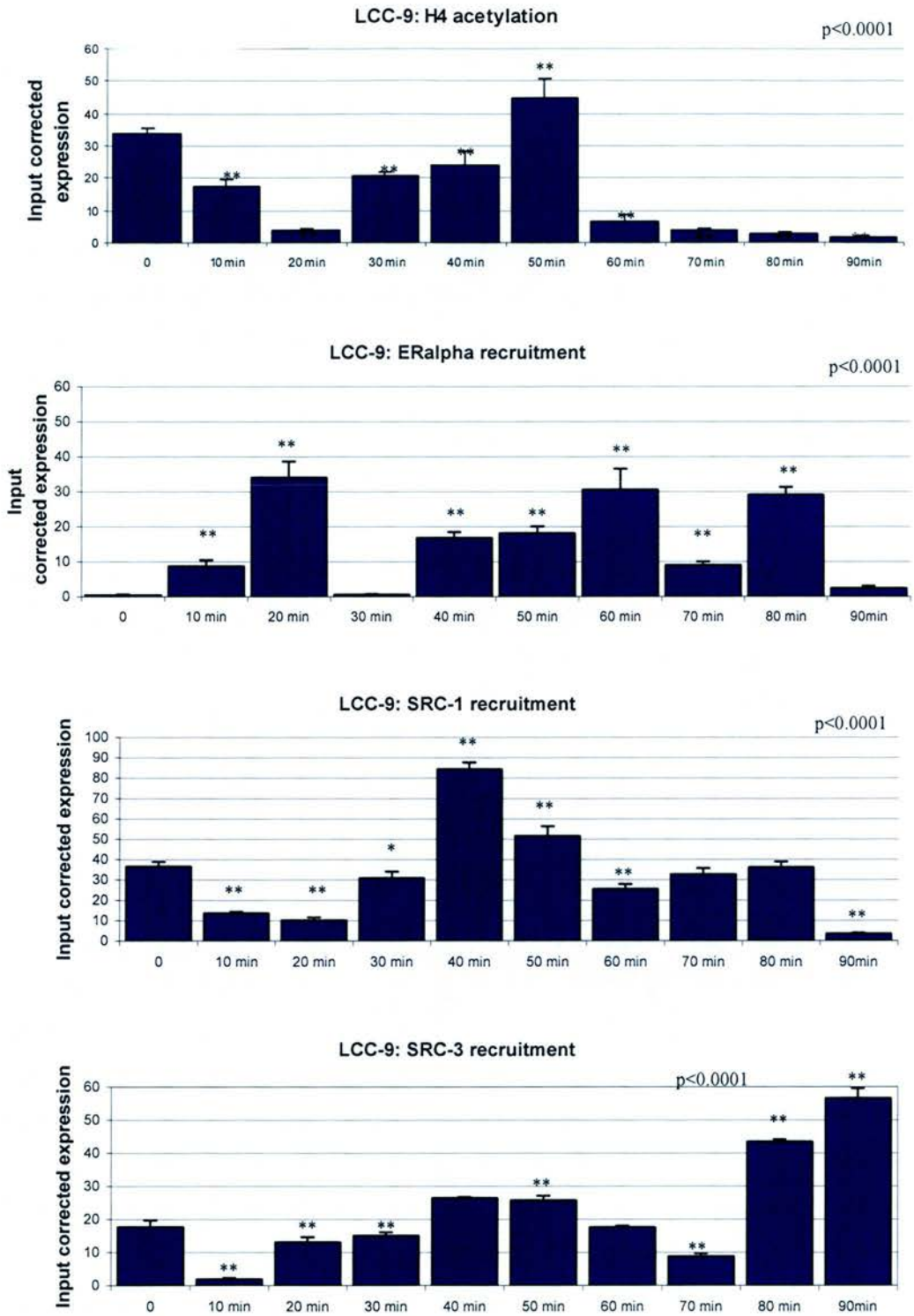
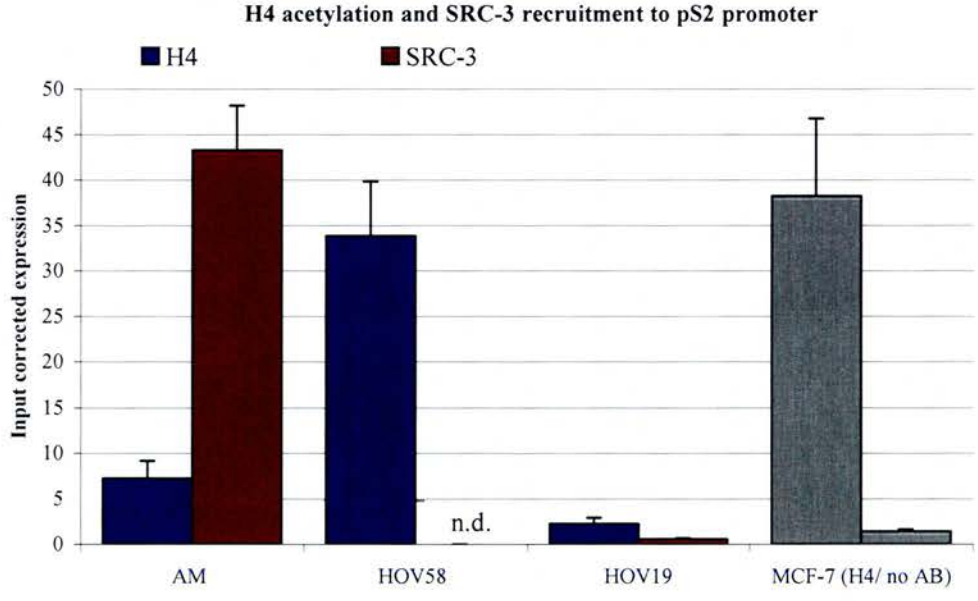


Figure 2.50: H4 acetylation and ERα/ SRC-1 recruitment to pS2 promoter in LCC-9 cells. Cells were plated in reduced serum (5%FCS in DMEM lacking phenol red) for 24h and treated with 10^{-7}M E_2. Chromatin fractions were prepared every ten minutes. Data shown are the result of a single collection. Each bar represents the mean of at least triplicate PCR analysis for each sample. Error bars = STD. Significant variance between 0h (no treatment) and time points determined by one-way ANOVA and Dunnets multiple comparison test where *=p<0.05, **=p<0.01.

2.5.6 ChIP using breast and ovarian tumour samples

To test the feasibility of ChIP analysis in clinical samples, H4 acetylation and coactivators SRC-3 recruitment were examined in a small selection of ER expressing human ovarian tumours (figure 2.51). Results revealed remarkable differences in acetylation as well as SRC-3 recruitment. Highest H4 acetylation in the sample designated HOV58 was comparable to levels detected in MCF-7 cells. ER protein content inversely correlated with tumour H4 acetylation. SRC-3 recruitment varied between non detectable levels and very strong expression in the sample designated AM.



	ER	PR
AM	59	NT
HOV 58	36	9
HOV 19	116	4

Figure 2.51: H4 acetylation SRC-3 recruitment to pS2 promoter in human ovarian tumours and controls (MCF-7 cells treated with 10^{-7}M E_2 for 40min). Data representative of two separate PCR runs. ER and PR status noted in table underneath (fmol/mg protein).

Summary of fold changes in cofactor recruitment to pS2 promoter

	SRC-1				SRC-3			
	10min	**20min**	**40min**	**80min**	**10min**	**20min**	**40min**	**80min**
MCF-7	+	-	-	+	+	++	+	++
LCC-1	+	+	+	+	++	+++	++	+++
LCC-9	--	--	+	-	--	-	+	+

Table 2.6: Summary of SRC-1 and SRC-3 recruitment to pS2 promoter in response to E_2 in MCF-7, LCC-1 and LCC-9 cells. Fold changes are indicated in comparison to time point 0h (no treatment). +++ = >50 fold increase; ++ = >5 fold increase; + = >1 fold increase; - = >0.5 fold decrease; -- = < 0.1 fold decrease.

2.5.7 Discussion

Studying chromatin regulation and the site of transcription is currently the centre of much research. This is based on the recognition that chromatin is not just a way of tightly packaging DNA but a site where complex signals are integrated to regulate gene transcription. Histone modification by acetylation facilitates DNA access to restriction enzymes and transcription factors and has been linked to transcriptionally active chromatin fractions (Li, Y-J. *et al.* 2004). Proteins such as the members of the p160 family with histone acetylation or deacetylation functions thus play a vital role in determining transcriptional output.

The technique of chromatin immunoprecipitation enables the detection and identification of factors involved in gene transcription *in vivo*. This method allows for a 'snap shot approach', and a detailed analysis of transcription sites. Activation of an oestrogen responsive gene pS2 via histone H4 acetylation was examined in oestrogen dependent MCF-7 cells and compared with oestrogen independent but responsive LCC-1 and resistant LCC-9 cells. Of particular interest was the involvement of ERα, the hormone's mediator, and recruitment of cofactors SRC-1 and SRC-3 known to be influenced by oestrogen and known to play a role in breast cancer. Using a detailed time course, transcriptional kinetics were examined in each of the cell lines. Differences in transcription factor participation and transcription complex assembly might help to elucidate ER-mediated gene transactivation in these tissues.

Much of the current knowledge of transcriptional activation of oestrogen responsive genes is based on *in vitro* work with MCF-7 breast cancer cells. It was

therefore essential to confirm these findings in MCF-7 cells. ChIP analysis demonstrated a cyclic pattern of H4 acetylation with coordinated recruitment of ERα as well as coactivators SRC-1 and SRC-3. The ER and both cofactors are recruited immediatly after hormone addition (see table 2.6). These results suggest the dynamic assembly of a transcription complex at the pS2 promoter involving the ligand-bound oestrogen receptor consistent with previously described models (Métivier, R. *et al.* 2003 and others). Transcriptional activation was less intense in the first of the two 45min cycles. Transcriptional activity was highly regulated by oestrogen. These results are supported by previous mRNA analysis where not only oestrogen inducibility was clearly demonstrated but also an accumulation of pS2 mRNA became apparent in the continued presence of the hormone.

Several researchers have reported immediate acetylation of H4 in response to oestrogen. An earlier study describes a 5-8-fold increase in acetylation within minutes for the pS2 as well as MYC and CTSD genes (Chen, H. *et al.* 1999). An acetylation peak reached at 1h was followed by a reduction. For pS2, promoter association with SRC-3 as well as ERα was reported. Acetylation of p160 proteins by p300/CBP was suggested to lead to cofactor dissociation. This suggested to lead to

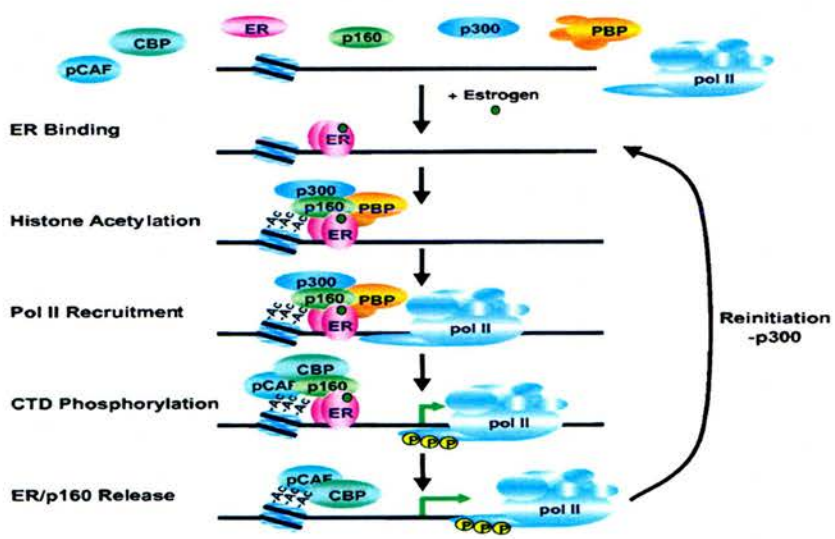


Figure 2.52: Dynamic model of ER mediated gene transcription (Shang, Y. *et al.* 2000).

cofactor dissociation. This gave the first indication against constitutive gene activation but in favour of a dynamic association and dissociation process to regulate gene transcription. A study comparing ER -positive MCF-7/T5 variant cells with ER

-negative MDA-MB-231 cells shows an increase in H3 and H4 acetylation in response to E_2 in MCF-7/T5 cells only highlighting the fact that this gene activation is ER dependent (Sun, J.M. *et al.* 2001). Others have presented results for oestrogen responsive genes showing weak ER binding in the absence of the ligand in contrast with strong enhancement once E_2 is added (Shang, Y. *et al.* 2000; Planas-Silva, M.D. *et al.* 2001; Zhang, H. *et al.* 2004). Studies have since revealed detailed kinetics of transcriptional activation in MCF-7 cells. Based on ChIP experiments over the course of 2h and detailed observations of sequential recruitment of transcriptional factors, a cyclic model of ER transcription was suggested for CTSD (Shang, Y. *et al.* 2000). In agreement with results in this experiment, acetylation does not return to baseline after an initial cycle. Two 45min cycles of transcription were proposed (see figure 2.52). Upon oestrogen addition, the ER binds to the promoter and actively recruits a histone acetylate transferase (HAT) containing p160-p300 and a PBP containing transcription complexes. Following histone acetylation, RNA polymerase II is recruited and subsequently phosphorylated. Transcription is now active. p300 is then replaced by CBP, also a histone acetylase, and p300/CBP associated factor pCAF brought in. Association of CBP leads to the release of p160 proteins via acetylation and dissemblance of the transcription complex including CBP and pCAF. All factors are now available for the following transcription cycle.

When examining such detailed dynamics of transcripiton, it is of concern that cells are harvested in different functional states particularly since large numbers of cells are needed for each precipitation. In this project, exact dynamics were not considered as important as the identification of transcription factors and hormonal regulation of their recruitment comparing different hormone responsive phenotypes. However, others have provided further insight into the kinetics of the transcription process utilizing α-amanitin cell cycle synchronized MCF-7 cells (Métivier, R. *et al.* 2003). Again, the role of the oestrogen receptor for pS2 transcription initiation in MCF-7 cells was explored and its involvement verified. A large array of additional transcription factors involved in active transcription were identified. These included histone methyltransferases such as CARM1, mediators such as TRAP150 and multiple additional general transcription factors such as TBP. An additional initial cycle was suggested to be transcriptionally silent. This cycle lasted 20min and

acetylating and dimethylating histones H3 and H4 were at a constitutive level. The recruitment of distinct HAT and histone methylation transferase (HMT) containing cofactors in the following two cycles leads to increased histone acetylation and gene activation. The authors not only confirm the sequential assembly of a transcription complex but also identify several different complexes. This would suggest that the rate of transcription is regulated by adaptation of individual cycles of a continuous gene transcription in response to a changing cell environment and emphasizes not only sequential but also combinatorial mechanisms of gene transcription. Depending on transcription factors present and recruitment to the promoter, accessibility of the gene as well as activation and repression or duration of transcription could be altered in a short space of time.

Such a mechanism is substantiated by the fact that most transcription factors were reported to be already present at the promoter in the absence of E_2 and were only further recruitment stimulated by its addition (Métivier, R. *et al.* 2003). This was also the case in the current experiment where H4 acetylation and recruitment of coactivators was detected at low levels in the absence of E_2. Other transcription factors have been shown to associate with the pS2 promoter without E_2. Low levels of acetylated H3 and H4 are maintained without E_2 alongside active binding of Sp1 family members Sp1 and Sp3 whose binding sites are found close to ERE sequences, in MCF-7 cells (Sun, J.-M. *et al.* 2005). Addition of oestrogen increases acetylation levels and reduces Sp1 but not Sp3 binding. In a previous study, the ER is suggested to alter the balance of HATs and HDACs such as SRC-1 or CBP (Sun, J.-M. *et al.* 2001). After ER localization to nuclear matrix on ligand binding, the receptor reorganizes already available transcriptional coactivators to increase the deacetylation rate. Together this underlines the theory of a dynamic transcription process where the promoter is in a 'state of readiness' as suggested by the authors.

The sequential and combinatorial transcription model provides the opportunity to apply the same mechanism as a general model to other gene promoters as well as tissue types. Each cycle can be continuously adapted to changing conditions such as binding sequences and spatial organisation within promoters and can involve different transcription factors. Dynamic recruitment of an ERα transcription complex has been demonstrated in ECC-1 and Ishikawa endometrial

carcinoma cells as well as the hepatocarcinoma cell line HepG2 (Shang, Y. *et al.* 2000 and 2002 (a); Barkhem, T. *et al.* 2002(a)). The corepressor SMRT potentiates transcriptional repression in a large transcriptional complex at the thyroid receptor (Guenther, M.G. *et al.* 2000). ChIP studies revealed a coordinated recruitment of SRC-2 and RNA polymerase II in parallel with histone acetylation for androgen receptor (AR) mediated PSA transcription, a gene coding for the prostate-specific antigen (Shang, Y. *et al.* 2002 (b)). Different transcriptional cofactors are recruited to the promoter of the PSA gene in response to agonist or antagonist bound AR association. The dynamics of transcription complex assembly are again found to be similar to ER mediated transcription though with different timing.

Much less knowledge has been accumulated about ER mediated transcription in hormone independent breast tissue. The results obtained here demonstrated that the pS2 gene is actively transcribed in a constitutive manner in LCC-1 and LCC-9 cells. Levels of transcription were much higher in oestrogen independent but responsive and tamoxifen independent LCC-1 cells as well as oestrogen and tamoxifen unresponsive LCC-9 cells. Oestrogen stimulation of pS2 transcription was small. This indicated a pS2 transcriptional mechanism different to MCF-7 cells where the ER is alternatively activated. ChIP analysis of H4 acetylation revealed a remarkably similar transcription pattern between MCF-7 and LCC-1 cells. Addition of E_2 led to cyclic stimulation of gene transcription. Interestingly, H4 acetylation in LCC-9 cells was also altered by the presence of E_2 although in a pattern opposite to that seen in MCF-7 or LCC-1 cells. An initial downregulation suggests an antagonistic effect on oestrogen. Next, involvement of the oestrogen receptor was examined and also revealed no obvious differences between hormone dependent and independent cell lines with clear oestrogen stimulation in ER recruitment remaining. Together, these results demonstrate active pS2 transcription involving ER binding regulated by E_2.

Whether the ER is activated directly by ligand binding, or indirectly through cross-talk to other growth factor pathways remains a subject of debate. Recruitment of the ER in oestrogen and tamoxifen sensitive MCF-7 cells but also oestrogen sensitive and tamoxifen resistant MCF-7/HER2-18 cells has been reported *in vitro* and *in vivo* (Shou, J. *et al.* 2004). In both models, ER activation took place via

phosphorylation of Ser-118 but only in the HER2 overexpressing MCF-7 variant MCF-7/HER2-18 did oestrogen and tamoxifen also induce phosphorylation of EGFR, HER2, Akt and ERK1/2 MAP kinases. This suggests an indirect path of ER activation where E_2 and tamoxifen activate alternative growth factor pathways, which then in turn activate the ER via phosphorylation. This could explain the remaining regulatory effect of E_2 on ER mediated gene transcription observed in hormone independent LCC-1 and LCC-9 models. Such cross talk leading to indirect ER activation has extensively been demonstrated for example by Ser^{118} phosphorylation through the EGFR or HER activated MAPK pathway as previously discussed (Kato, S. *et al.* 1995; Font de Mora, J. *et al.* 2000). With alternative receptor activation in place, ER function might not be ER -ligand -binding dependent. Elevated levels of pS2 expression in the absence of the hormone have been reported in ERαHA cells, a tet-inducible ERα overexpressing MCF-7 cell line (Fowler, A.M. *et al.* 2004). Moreover, pS2 expression levels in these cells were comparable to expression levels in uninduced control cells in the presence of E_2. Traditionally, ER activation has been linked to transactivation domains AF-1 and AF-2, the former in a ligand –dependent manner, the latter constitutive. In this model, pS2 transcription is AF-2 independent but AF-1 dependent and ERα is found to be strongly bound to the pS2 promoter despite the absence of the hormone. This transcriptional activation could not be linked to cross-talk to ERK/MAPK pathway given that MAPK signalling factors Erk and Elk1 were not activated. Most interestingly, ERαHA cells proliferate in the absence of oestrogen which compares with a static level of proliferation in MCF-7 cells. Together, results indicate that an increase in cell growth in hormone independent phenotypes might in part be due to increased unliganded ERα gene expression. This observation correlates with LCC-1 and LCC-9 models where cell growth is similar in the presence and the absence of E_2 and ERα mRNA and protein expression is elevated compared to hormone sensitive MCF-7 cells.

To explain increased ERα AF-1 function, it is speculated that receptor abundance might stabilize an active receptor confirmation and increase recruitment and association with various transcriptional cofactors such as the p160 proteins (Fowler, A.M. *et al.* 2004). This would assign such coactivators a critical role in

ligand independent gene transcription. ChIP analysis presented here does provide evidence for this suggestion. Coactivators SRC-1 and SRC-3 are recruited to pS2 promoter in both, LCC-1 and LCC-9 cells. The addition of oestrogen leads to an initial increase in SRC-1 and SRC-3 promoter association although only in LCC-1. SRC-1 and SRC-3 recruitment in LCC-9 cells is initially decreased upon hormone treatment as to suggest that both coactivators are strongly associated with the promoter but easily detached once E_2 is present (see comparison in table 2.6). These coactivators might be associated with other cofactors during the transcriptionally inactive gene and the absence of E_2. Recruitment of ERα in response to E_2 arrival may trigger their release to initiate the assembly of transcription complexes activating gene transcription. The levels of promoter occupancy for SRC-3 in LCC-1 and both p160 proteins in LCC-1 and LCC-9 cells were significantly higher than levels observed in parental MCF-7 cells. Previously, protein analysis showed that SRC-1 was undetectable in MCF-7 cells but present in LCC-1 and LCC-9 cells (chapter 2.4). To support this key role for p160 coactivators, SRC-1 and other cofactors have been shown to enhance ligand-independent ER mediated gene transcription (Kalkhoven, E. *et al.* 1998; Lavinsky, R.M. *et al.* 1998). More specifically, SRC-1 and CBP enhance AF-1 activity of the unliganded ERα to potentiate pS2 gene transcription (Duterte, M. and Smith, C.L. 2003). Coactivator action is due to interaction with phosphorylation sites in the receptor A/B domain as well as classical recruitment through the LBD in the E section suggesting a possible cofactor enhanced synergism between the two receptor domains to mediate ER transactivation (Onata, S.A. *et al.* 1998; Benecke, A. *et al.* 2000).

Several studies propose models where cofactors serve as links not only between the receptor and other transcription factor but also between different regions within the promoter or surrounding the promoter. The androgen receptor is recruited to the promoter of the PSA gene as well as an enhancer region located 4kB upstream from the starting site (Shang, Y. *et al.* 2002 (b)). Coactivators such as SRC-2 and CBP and RNA pol II are also recruited to both regions. Cofactor recruitment and histone acetylation dynamics were found identical between the two sequences and PSA mRNA expression detected to correlate with this timing. In addition, SRC-2 mutation studies have shown that the coactivator bound to both, the A/B domain and

the LBD can also directly enhance AR activity by bridging the two receptor domains (Shen, H.C. *et al.* 2005). ER mediated activation of the pS2 gene in HepG2 carcinoma cells is dependent on cooperation between ERE and AP-1 regions within the promoter (Barkhem, T. *et al.* 2002(b)). Underlined by the finding that HAT containing cofactors such as CBP/p300 do not directly interact with the receptor, it seems plausible to assume that transcription complexes formed at different regions of the gene are physically brought together to share coactivator proteins to stabilize and facilitate transcription through a large coactivator complex. Transcriptional response and p160 cofactor potentiation might be dependent on the ERE sequence itself resulting in distinct receptor confirmations and differential cofactor recruitment, but also the relative position of cooperating elements of the gene. For example, decreasing the distance between the pS2 ERE and AP-1 site has been reported to increase transcription (Barkhem, T. *et al.* 2002 (a)). In the same study, SRC-1 and SRC-2 mRNA expression was found to be similar. However, distinct functionality was revealed for the two proteins. This could help to explain observations in this project where increased pS2 p160 protein recruitment was observed but not reflected in mRNA expression. While both, SRC-1 and SRC-2, potentiate pS2 transcription via ERE and AF-1 motifs, AF-1 deletion resulted in differentially potent gene activation by SRC-1 and SRC-2. SRC-2 functionality appears less dependent on AF-1. As discussed during earlier chapters, extensive evidence does suggest p160 proteins exhibit distinct functionality such as differences in ERα binding affinity between SRC-1 and SRC-2 (Hall, J.M. *et al.* 2002).

The concept of chromatin dynamics and the large number of identified factors involved in ER mediated gene transcription provides insight into the complexity of transcription. ChIP analysis in this study supports a transcriptional mechanism with great potential for adaptation according to the gene and its environment. This posttranslational gene regulation might explain the differential hormone response in breast cancer tissue, but it is also likely that general features apply to most hormone responsive genes in eukaryotic cells.

Chapter 3: Conclusions and future directions

3.1 Summary

Oestrogen plays a vital role in the growth and development of the normal breast but can also mediate proliferation into malignant phenotypes. A major problem in breast cancer development concerns the changes in phenotype from hormone dependent to independent growth. Understanding the precise mechanism of E_2 action is the basis for advances in endocrine therapies. To help elucidate the mechanism of hormone resistance this study examined the regulation of E_2 mediated target genes comparing breast cancer cell lines with differential phenotypes regarding hormone responsiveness. One such gene was selected to investigate transcriptional activation in response to E_2.

A breast cancer cell line model was chosen where classically E_2 and tamoxifen sensitive MCF-7 cells serve as a common parent line for three MCF-7 variant cell lines with distinct phenotypes; LCC-1 cells demonstrated a modest E_2 sensitivity and tamoxifen resistance while LCC-1, LCC-9 and LY2 cells showed E_2 and tamoxifen resistance (figure 3.1). This model therefore represents diverse human breast cancer phenotypes and was appropriate to explore differences in hormone regulated gene transcription.

The parental MCF-7 cell line clearly differs from all the variant cell lines examined by its oestrogen and tamoxifen sensitivity with respect to cell growth. The analysis of ERα and PR expression as well as a series of oestrogen target genes

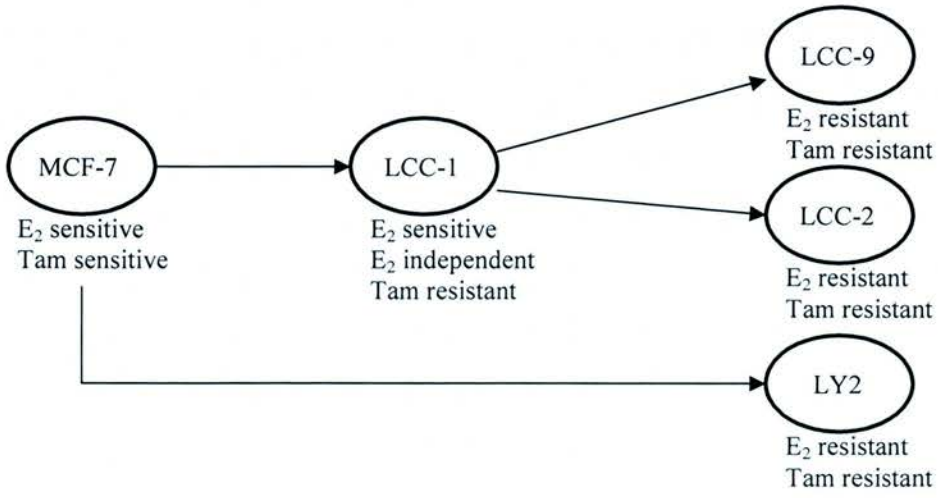


Figure 3.1 Summary of results: Growth characteristics of MCF-7 and MCF-7 variant cells.

showed that E_2 has profound effects on mRNA and protein expression of these genes. In the absence of E_2, ERα and PR mRNA were expressed at relatively low levels. E_2 greatly reduced ERα and increased PR protein expression. The expression of pS2, CTSD and MYC mRNA were increased by E_2 although the most dramatic stimulation was observed for pS2 after 48h of hormone exposure (663 fold). Coactivator and corepressor mRNA were found to be expressed at comparable levels with relatively small effects being modulated by E_2 and tamoxifen with few exceptions, for example, a strong stimulation of RIP140 by E_2. However, protein expression revealed marked variations. While coactivator SRC-1 protein could not be detected, the other p160 family members, SRC-2 and SRC-3, were expressed strongly and unaffected by the presence of E_2 or tamoxifen. Variations in multiple protein band expression of RIP140 in different treatment groups suggested posttranslational changes. A detailed analysis by ChIP showed cyclic H4 acetylation, indicative of active pS2 gene transcription, as well as cyclic recruitment of coactivators SRC-1 and SRC-3 and the recruitment of ERα to the promoter of pS2 in response E_2 as previously reported (Shang, Y. *et al.* 2000; Métivier, R. *et al.* 2003). In the absence of E_2, the level of H4 acetylation and the level of ERα and cofactor recruitment is low.

All variant LCC as well as LY2 cell lines share a characteristic complete E_2 independence with respect to cell proliferation. Uniquely, the LCC-1 cell line was shown to be E_2 independent but responsive to the hormone. A small increase in proliferation was observed in the presence of E_2. In common with the other variant lines, ERα and PR mRNA were expressed at significantly higher levels in LCC-1 cells. Compared to MCF-7 cells, ERα protein expression was much higher but like the parent line, was reduced to almost undetectable levels in the presence of E_2. The expression of PR protein was high in the presence of E_2; tamoxifen also increased expression of PR protein in LCC-1 cells. This tamoxifen induced expression was not detected in MCF-7 cells. Most strikingly, pS2 mRNA expression was considerably higher in the absence of E_2 in LCC-1 cells and a comparably small stimulation was observed once E_2 was added. The expression of cofactors RIP140 and NCoR mRNA were particularly strong in LCC-1 cells. A short term increase in mRNA expression was observed for all cofactors but SRC-3. A strong induction by E_2 was also

observed for SRC-1 and RIP140 protein expression. ChIP analysis revealed varying acetylation and recruitment patterns to the pS2 promoter. H4 acetylation was found to be in a 'ready state' in the absence of the hormone. Acetylation, ERα and cofactor recruitment were induced in response to E_2. While the recruitment pattern for both coactivators was comparable, the level of recruitment was markedly higher, particularly for SRC-1, compared to MCF-7 cells.

LCC-2 and LCC-9 cells showed many similarities in addition to their common resistance to E_2 and tamoxifen. As with LCC-1 cells, pS2 and MYC mRNA were expressed at high levels compared to MCF-7 cells in LCC-2 and LCC-9 cells. E_2 mildly increased whereas tamoxifen had no effect on pS2 mRNA expression in both cell lines resulting in constitutive expression of the gene. Comparable levels of cofactor mRNA were generally decreased by E_2 exposure between 3h and 6h. Differential expression of cofactor protein was detected also in response to E_2 and tamoxifen. E_2 increased the expression of SRC-1 and SRC-3 protein but reduced the strong baseline expression of SRC-2 in LCC-2 cells. SRC-1 and SRC-3 were generally expressed at very low levels in LCC-9 cells. LCC-2 and LCC-9 cells could be clearly distinguished by their ERα but also PR mRNA and protein expression. Basal expression of ERα mRNA was high compared to MCF-7 cells in both variant lines and reduced by E_2. However, the basal level of ERα protein detected in LCC-2 cells was considerably stronger than in LCC-9 cells. While E_2 reduced protein levels in both cell lines, tamoxifen, which has no effect on protein expression in LCC-2 cells, also reduced ERα protein in LCC-9 cells. PR mRNA was particularly strong in LCC-9 cells compared to MCF-7 but also LCC-2 cells. The most remarkable variations occurred on the PR protein level where a uniformly strong band was revealed across all treatment groups in LCC-9 cells, whereas PR protein in LCC-2 cells could only be detected in the presence of E_2. ChIP analysis of the pS2 promoter in LCC-9 cells showed a distinct pattern of H4 acetylation. Initially high levels of acetylation were decreased after E_2 exposure before recovery into an acetylation pattern different to MCF-7 cells. Also in contrast to MCF-7 cells, coactivators SRC-1 and SRC-3 were found to occupy the promoter in the absence of E_2 and recruited following E_2 exposure in much higher levels. ERα recruitment followed a pattern similar to MCF-7 cells.

Unlike LCC-2 and LCC-9, LY2 cells are direct descendants from MCF-7 cells and were derived as a separate branch from all LCC lines (see figure 3.1.1). Nevertheless, growth phenotype, ERα and PR as well as E_2 target gene and cofactor expression are most similar to LCC-9 cells. Like LCC-9 cells, LY2 cells expressed higher ERα mRNA than MCF-7 and strong PR mRNA. E_2 as well as tamoxifen reduced ERα protein to non -detectable levels. PR protein expression in LY2 cell was low irrespective of E_2 or tamoxifen presence which stands in contrast to the strong expression of the receptor protein in LCC-9 cells giving LY2 cells a unique characteristic. pS2, CTSD and MYC expression levels were most similar to LCC-9 but also LCC-2 cells with generally small effects produced by E_2 and tamoxifen. p160 coactivator mRNA was expressed at differing levels. SRC-3 expression was particularly low. Similar to LCC-9 cells, SRC-1 and SRC-3 protein was found to be scarcely expressed whereas SRC-2 protein expression was particularly strong.

3.2 Conclusions

The key results of this project are summarized in figure 3.2. The analysed cell lines exhibit distinctively different phenotypes. Where MCF-7 cell growth was shown to be highly dependent on the presence of E_2, LCC-1 cells exhibited E_2 independence but cell proliferation remained sensitive to the hormone whereas LCC-2, LCC-9 and LY2 cells were E_2 independent and were completely insensitive to E_2. (figure 3.2 (C)). The comparison of ERα transcription and translation between MCF-7 and variant cell lines did not produce any indications that loss of expression or function of the receptor determines the phenotype of these cells. However, it is obvious that the expression and the modulation of E_2 and tamoxifen on ERα, PR and several ERα target genes differs between phenotypes (figure 3.2 (B)). Most markedly, expression for all analysed E_2 target genes was strongly E_2 inducible in MCF-7 cells. In contrast, E_2 (and tamoxifen) showed no effect or relatively small increasing or decreasing effects in all MCF-7 variant cell lines. This implies profound mechanistic changes in E_2 modulated gene expression between hormone sensitive and hormone insensitive breast cancer cells.

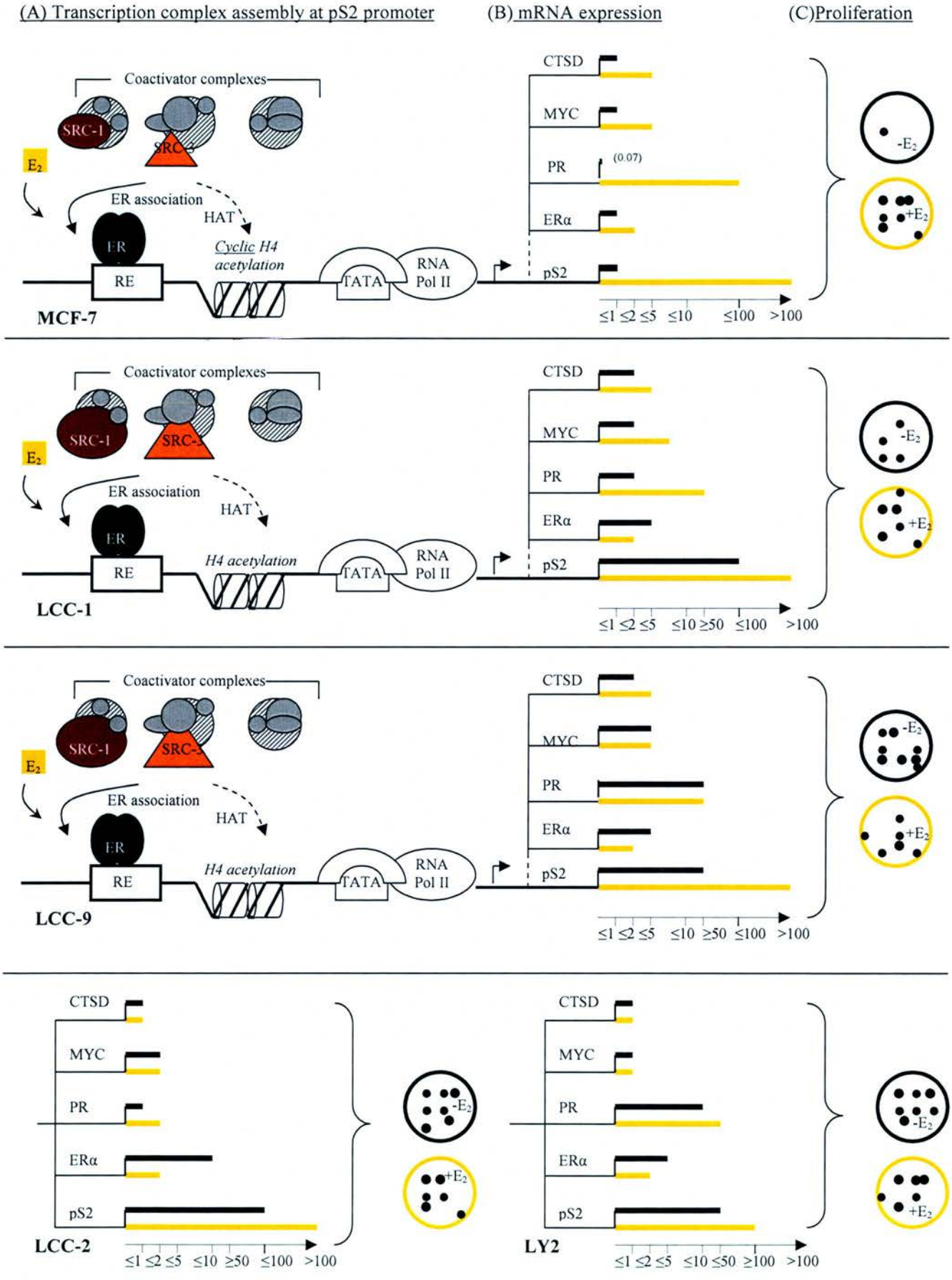


Figure 3.2.: Summary of key results comparing MCF7 with MCF-7 variant cell lines: (A) Transcription complex assembly as observed by ChIP. H4 acetylation and SRC-1/3, ERα recruitment to pS2 promoter. RE=response element. ●⊘ other cofactors. See text for details. (B) mRNA expression of E_2 target genes at 48h with (—) and without (—) E_2. Scale corresponds to gene/actin ratio as observed by RT-PCR. (C) Cell proliferation with (o) and without (**o**) E_2 at day 6. ● represents ≥1000 (x10^4) cells.

ChIP analysis suggested cell-type specific dynamics of H4 acetylation and involvement of ERα and p160 coactivator in transcription complex assembly irrespective of the cells growth characteristics with respect to hormone sensitivity (figure 3.2 (A)). Acetylation of histone H4, indicative of active pS2 gene transcription, in response to E_2 was observed in all three cell lines and confirmed to be of a specific cyclical pattern in MCF7 cells (Shang, Y. *et al.* 2000). However, in LCC-1 and LCC-9 cells H4 acetylation appears at a 'steady state' in the absence of E_2 before further recruitment in a distinct albeit unpredictable pattern after hormone addition. In LCC-1 and LCC-9 cells, higher levels of coactivators recruitment (indicated by proportionally larger symbols for SRC-1 and SRC-3) were detected. hormone sensitive and hormone insensitive breast cancer cells or changes in basal (non-oestrogen stimulated) conditions. Further, levels of cofactor recruitment particularly SRC-1 did not change between LCC-1 and LCC-9 cells, yet the dynamics of recruitment were altered. E_2 appears to facilitate further recruitment as the promoter-bound coactivator is initially removed from the site and re-recruited to high levels by E_2.

In all cell lines, transcription is likely to take place by assembly of several transcription complexes involving a multitude of transcriptional cofactors as suggested by Métivier, R. *et al.* 2004 and others. Sequential and combinatorial recruitment of HATs, HMTs and other mediator proteins into complexes, mediates posttranslational histone modification and factors of the basal transcription machinery including RNA pol II initiate active gene transcription. The differential phenotypes appear to be based on an altered mechanism of ERα mediated gene transcription. These findings are consistent with evidence of changed dynamics of histone acetylation and transcription complex assembly.

3.3 Future directions

Considerable progress has been made in understanding oestrogen mediated gene transcription. However, the precise mechanism remains elusive particularly with respect to changes that occur during the acquisition of hormone resistance.

The results described here are based on detailed analysis of a single gene. It will be important to determine which other genes play an important role in orchestrating cell proliferation in response to E_2. Detailed analysis of MYC or CTSD using the ChIP technique might confirm the cell-type specific transcription complex assembly established for the pS2 promoter and help to determine potential promoter specificity. High levels of cofactor expression has previously been suggested to be indicative of tamoxifen resistance (Osborne, C.K. *et al.* 2003). Cofactor knockout experiments using RNAi would lead to further insight into the differential role of individual cofactors in transcription complex assembly and reveal functional significance determining the cells' growth response to E_2 and tamoxifen. Knockout of multiple transcriptional cofactors could indicate potential functional interdependence or redundancy characteristic for specific phenotypes. Such experiments also apply to involvement and functionality of ERα. The correlation of cofactor expression to oestrogen and/or tamoxifen resistance might provide the opportunity for SRC-1 or SRC-3 to serve as diagnostic markers. This could identify a subgroup of ER positive patients who fail to respond to endocrine therapy or present with an acquired tamoxifen resistance during treatment.

The discovery of enzyme families that modify chromatin structure to facilitate gene transcription created new therapeutic approaches (Spotswood, H.T. and Turner, B.M 2002). The selective inhibition of HAT and HDAC activity carrying enzymes might prevent abnormal cell proliferation. Analysis of the effect of HAT and HDAC inhibitors on pS2 transcription would provide additional information on the mechanism of histone acetylation and deacetylation to regulate gene transcription.

ChIP analysis of cells treated with antioestrogens such as tamoxifen would explore the potential absence of coactivators such as the p160 family at the pS2 promoter in E_2 dependent MCF-7 cells as suggested in previous publications (Shang,

Y. *et al.* 2000). Promoter occupancy in the presence of tamoxifen in breast cancer cells with an acquired hormone independence is currently being studied in our laboratory.

Finally, preliminary results have shown that ChIP can be applied to examine breast cancer tumour material. Analysis of pS2 promoter occupancy in response to E_2 in a panel of tumour material might offer confirmation of the differential role of coactivators in E_2 in insensitive cells and help identify other factors involved in the acquisition of hormone independence.

4. Chapter: Material and Methods

4.1 Materials

Materials are listed by technique. All chemicals are from Sigma unless otherwise stated. All laboratory plastics are from Nuncleon, Life Technologies unless otherwise stated. Primers and antibodies are listed in the relevant RT-PCR, ChIP and Western blot method sections.

4.1.1 Cell culture

(i) Cell lines

All cell lines were kindly provided by Dr.Robert Clarke, V.T.Lombardi Cancer Research Center, Georgetown University Medical School, Washington, D.C., USA. The MCF-7 cell line was originally established by Dr. Herbert Soule who obtained the cells from a pleural effusion taken from a patient with a breast carcinoma (Soule, H.D. *et al*, 1973). LCC-1, LCC-2, LCC-9 and LY2 cells are sublines of MCF-7 cells selected *in vivo* and *in vitro* for their different oestrogen and antiestrogen responsiveness (Brünner, N. *et al.* 1993a, 1993b, 1997; Bronzert, D.A. *et al.*1985). MDA-MB-231 cells were obtained from the American Type Culture Collection.

(ii) Tissue culture reagents

Cryovials	Nuncleon, Life Technologies
Tissue culture flasks	Nuncleon, Life Technologies
Petri dishes and well trays	Nuncleon, Life Technologies
Waterbath	Grant Instruments Cambridge Ltd.
Trypsin	Gibco BRL, Life Technologies
Phosphate Buffered Saline (PBS)	Gibco BRL, Technologies
Foetal calf serum (FCS)	PAA limited
Penicillin/Streptomycin	Gibco BRL, Life Technologies
DMEM growth media +/- phenol red	Gibco BRL, Life Technologies
E_2	Sigma, Poole, Dorset, UK
TAM	Sigma, Poole, Dorset, UK

4.1.2 RNA extraction and RT-PCR

RNA isolation kit	Qiagen Ltd., Crawley, UK

Tri-Reagent	Sigma, Poole, Dorset, UK
DNase digestion	Qiagen Ltd., Crawley, UK
RT-PCR kit (SYBR®Green system)	Qiagen Ltd., Crawley, UK
PCR machine	Rotorgene RG-3000 Corbett Research
PCR analysis software	Corbett Research Version 6.0 (Build 25)
Centrifuges: Biofuge *pico/fresco*	Sorvall® Heraeus

4.1.3 Western blotting

Membranes	Immobilon-P
Chemiluminescence Western Blotting kit	Boehringer Mannheim
Signal West Femto Maximum Sensitivity Substrate	Pierce
Photographic paper: Hyperfilm	Amersham Biosciences UK Limited
Protein concentration assay kit	Bio-Rad Laboratories Ltd.
Tween 20	Bio-Rad Laboratories Ltd.
Heat block: DRIBLOCK®DB-2A	TECHNE
Protein ladders: 6-175kDa	NEB Biomarkers
4-250kDa	Invitrogen, Life technologies
Electrophoresis tank	Bio-Rad Laboratories Ltd.
Power Pack: Power PAC 3000	Bio-Rad Laboratories Ltd

4.1.4 ChIP assays

Protease inhibitor tablets	Roche Diagnostics Ltd.
Salmon Sperm DNA	Invitrogen, Life technologies
Protein G agarose beads	Roche Diagnostics Ltd.
DNA purification kit	Qiagen Ltd., Crawley, UK
PCR kit (SYBR®Green system)	Qiagen Ltd., Crawley, UK
DNA ladder: 500bp-12kB	Invitrogen, Life technologies
100bp-1500bp	Invitrogen, Life technologies
MassRuler^{TM} DNA ladder	Fermentas Life Sciences
Sonicator	Soniprep 150, MSE
Rotator	LABINCO

4.2 Methods

4.2.1 Cell culture

(i) Routine Culture of cell lines

MCF-7 cells were grown in phenol red containing DMEM supplemented with 10% foetal calf serum (FCS) and 1% penicillin/ streptomycin (complete medium). LCC-1, LCC-2, LCC-9 and LY2 cells were routinely kept in phenol free DMEM supplemented with 5% dextran activated charcoal stripped FCS (DCC), 1% penicillin/ streptomycin and 2mM Glutamine (reduced medium). All cells were grown at 37°C in 5% CO2 in a humidified incubator. Media was changed every 2-3 days.

(ii) Cell harvesting

Cells were grown in monolayers to 80% confluence in 75 or 175cm^2 flasks. Confluent flasks were washed in PBS pH 7.3 to remove traces of serum and detached by incubation at 37° in 1-3ml trypsin for 5-10min. Trypsin was inactivated by the addition of serum- containing media, and cells were pipetted up and down to produce a single cell suspension.

(iii) Cell storage and recovery from liquid nitrogen

Cells to be stored in liquid nitrogen were trypsinized as above and pelleted for 8min at 1800rpm. Supernatant was removed and cells were resuspended in 3-6ml of freezing media (10% DMSO in 90% FCS). Cells were transferred to cryovials and frozen stepwise (30min 4°C, 1-2h -20°C, overnight -80°C) before transfer to a liquid nitrogen tank.

Upon recovery, cells were defrosted in a 37°C waterbath for 1 min and centrifuged as described above to obtain a cell pellet for resuspension in medium. Cells were allowed to attach to the flask surface overnight before media was changed to remove dead cells.

(iv) Cell counting

To determine appropriate cell numbers for experimental set up, cells were trypsinized and centrifuged as above. The pellet was resuspended in 10ml media and suspension syringed to break up any clumps. Cells were counted using a haemocytometer and diluted appropriately for seeding.

(v) Dextran activation and charcoal stripping of serum

To remove enrichment factors from foetal calf serum for growth experiments, a charcoal mix was prepared consisting of 5g charcoal and 25mg dextran T70 in 50ml destilled H_2O and stirred overnight at 4°C. On the following day, 1 litre of serum was heat inactivated at 56°C for 30min. The serum was incubated with 2000U of sulphatase at 37°C for 2h and the pH adjusted to 4.2 using 2M HCl. The charcoal mix was added to the serum and left stirring overnight at 4°C. To remove the charcoal, the mix was centrifuged at 10,000 rpm for 30min at 4°C, the supernatant separated from the pellet and the pH readjusted to 4.2. A second prepared charcoal mix was added to the serum for a further 24h incubation at 4°C. To remove the charcoal, centrifugation as in the previous removal was carried out. A second centrifugation was added to remove any residual charcoal from the serum. The pH was adjusted to 7.2 using 2M NaOH. The serum was filter sterilised and aliquots stored at –20°C.

(vi) Growth assays

To determine the effects of 17β-oestradiol and tamoxifen on cell proliferation, cells were harvested by trypsinisation and plated in 6 well plates at a density of $2x10^4$ cells per well. MCF-7 cells were seeded in DMEM containing 10% FCS for 24 h. The media was changed to reduced DMEM with 5% DCC for 48 h. The media was then replaced with experimental media with or without 10^{-9} M E_2, 10^{-6} M tamoxifen or both. This was designated Day O. LCC-1, LCC-2 and LCC-9 cells were seeded in reduced DMEM containing 5% DCC FCS at the same density. Experimental media was added after 24h, day 0 of the experiment. For all cells, media was changed every two days. Cell counts were estimated using a Coulter Counter on Days 0,2,4 and 6. Cells were washed in 1ml warm PBS pH7.3. PBS was removed from the cells and set aside. Cells were harvested from wells in 200µl trypsin for 8min at 37°C. After addition of 1ml of media to each well, the suspension was removed and added to the PBS set aside previously. Cell pellets were collected by centrifugation for 4min at 1600rpm. Cells were resuspended in 1ml of growth media and syringed to break up

any aggregates before counting 200µl of the suspension in 9.8ml sodium chloride (0.9%) on a Coulter Counter.

4.2.2 RNA detection

(i) Cell seeding

MCF-7 cells were seeded in T175 flasks in normal DMEM with 10%FBS for 24 h. Cells were then washed twice in PBS and media added to reduced DMEM with 5% DCC for 48 h. The media was then replaced with experimental media with or without 10^{-9} M E_2, 10^{-6} M Tam or both. LCC1,LCC2 and LCC9 cells were immediately seeded in reduced DMEM with 5% DCC. Experimental media was added after 24h. All cells were exposed to experimental media for 48h by which time they had reached about 80-90% confluence.

(ii) RNA extraction

Extraction of total RNA from whole cells was performed using Tri-Reagent (Sigma, Poole, Dorset) as per manufacturer instructions. Media was removed from the culture flasks and Tri-Reagent added to the cells (1ml/ 25cm^2 flask surface). Cell lysates were triturated and transferred into 1.5ml Eppendorf tubes. Tri-Reagent was added (125µl chloroform/ 1ml Tri-Reagent), samples thoroughly vortexed and then centrifuged at 12 000rpm for 15min. The aqueous phase was carefully transferred to a new Eppendorf and 200µl isopropanol/ml was added and the samples briefly vortexed. After a 5min incubation at room temperature, the samples were centrifuged at 12 000rpm for 8min at 4°C. The resulting pellet was washed with 75% ethanol by vortexing and subsequent centrifugation at 7500rpm for 5min at RT, the pellet air dried for several minutes and resuspended in 50µl ultrapure water. From this point, samples were kept on ice throughout further processing. RNA concentration was measured using 5µl of the suspension in 995µl of ultrapure water in a spectrophotometer.

(iii) Reverse Transcription –Polymerase Chain Reaction

The QuantiTect™SYBR®Green system was used according to the manufacturers instructions for one step RT-PCR in a total of 15µl reaction volumes including 10µM each primer and 40ng RNA. Real Time cycler conditions were RT: 50°C for 30min; PCR: initial activation 95°C for 15min; followed by 40 cycles of denaturation 94°C for 15sec, annealing 57°C for 30sec, extension 72°C for 30sec; and a final extension of 72°C for 60 sec. Primer sequences utilized in this experiment were designed using Primer 3 (Whitehead Institute for Biomedical Research, http://www.genome.wi.mit.edu/cgi-

bin/primer/primer3_www.cgi) and synthesized by Cancer Research UK, Clare Hall, South Mimms, UK. Product sizes were verified by visualisation on agarose gels (figure 4.1). Sequences, NCBI accession codes and product sizes are listed in table 4.1.

Table 4.1 Primers used for RT-PCR

Target mRNA	Accession code	Primer sequence	Product size
SRC-3	AF012108	F5'CCC TTT TAT CTA CTC TGT CAT C3'	387bp
		R5'CCA GAT GTA GAG GAG GAG AC3'	
CTSD	NM001909	F5'CCC GAG GTG CTC AAG AAC TA3'	195bp
		R5'TCA CGT AGG TGC TGG ACT TG3'	
ERα	NM000125	F5'CCA CCA ACC AGT GCA CCA TT3'	107bp
		R5'GGT CTT TTC GTA TCC CAC CTT TC3'	
ERβ	NM001437	F5'AGA GTC CCT GGT GTG AAG CAA G3'	143bp
		R5'GAC AGC GCA GAA GTG AGC ATC3'	
MYC	NM002467	F5'TTC GGG TAG TGG AAA ACC AG3'	201bp
		R5'AGC AGC TCG AAT TTC TTC CA3'	
NCoR	NM006311	F5'AAA GTG TGG AGA CCC AGG TG3'	151bp
		R5'ACC CTC ACT TCA ACG TCC AC3'	
p300	BC053889	F5'CTT TCC CAG CCA GCT GTA AAG3'	382bp
		R5'CGG TAA AGT GCC TCC AAT GT3'	
PR	NM000926	F5'GTC AGT GGG CAG ATG CTG TA3'	193bp
		R5'AGC CCT TCC AAA GGA ATT GT3'	
pS2	NM003225	F5'TTG TGG TTT TCC TGG TGT CA3'	208
		R5'CCG AGC TCT GGG ACT AAT CA3'	
REA	AF150962	F5'CGA AAA ATC TCC TCC CCT ACA3'	396bp
		R5'CCT GCT TTG CTT TTT CTA CCA3'	
RIP140	NM003489	F5'CGG AAG AGG CTG TCT GAT TC3'	199bp
		R5'AGG GCA TAT CCT TGC TCC TT3'	
SMRT	NM006312	F5'AAG TCC ATC CTC ACG TCC AC3'	200bp
		R5'AAG CAC ACT GGG TCT CTG CT3'	
SRC-1	NM147223	F5'CAT GCT TAT GAG GCA GCA AA3'	266bp
		R5'ATT CCA GTG CCA AAC TGT C C 3'	
SRC-2	NM006540	F5'TCT GGA TAC CAG CAC CAT GA3'	198bp
		R5'GCA ACA AGA GTG CCA TCA GA3'	
β-actin	BC013835	F5'CTA CGT CGC CCT GGA CTT CGA GC3'	385 bp
		R5'GAT GGA GCC GCC GAT CCA CAC GG3'	

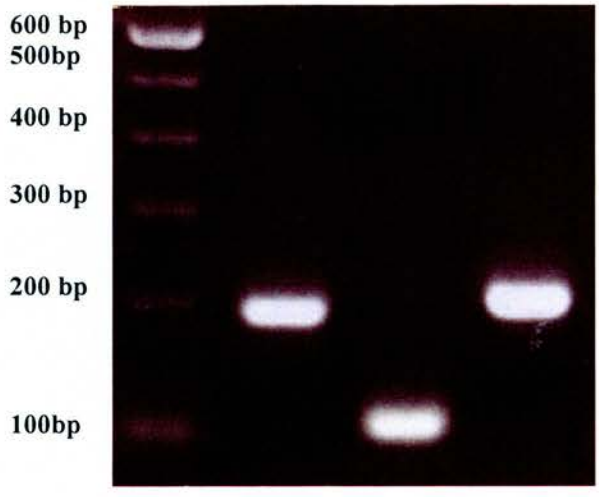


Figure 4.1: Size check for products of specific primers utilized in mRNA analysis. A representative experiment is shown.

(iv) Statistical analysis

Statistical assistance for these studies was provided by Dr. Robert Rush of Cancer Research UK. The following equation was used in all mRNA analysis experiments to calculate the propagation of errors:

$$\Delta z = z \left(\left(\frac{\Delta x}{x} \right)^2 + \left(\frac{\Delta y}{y} \right)^2 \right)^{1/2}$$

In this equation, z is the mean of the actin corrected sample value; x is the mean sample value; y is the mean actin value; and Δx and Δy are the corresponding standard deviations between the triplicate values that make up the means. This calculation therefore takes both standard deviation of actin as well as the standard deviation of the generated sample value into account. To determine statistically significant variance between all treatment groups, the ANOVA test was employed in all mRNA experiments. The overall p-value is noted on each graph. If a significant variation had been established, a *post hoc* test, the Dunnet comparison test, was employed to identify where differences occurred and compare each of the groups to the matched untreated control group. This statistical significance was marked with

asterisks. In some cases groups were compared with one another on an individual basis using the Tukey/ Newman Keuls multiple comparison test.

4.2.3. Protein detection

(i) Protein extraction

Cells were grown and treated in T175 culture flasks as described under RNA cell seeding. To isolate protein, cells were washed with ice cold PBS and lysed for 10 min on ice in 750µl/ flask lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150M NaCl, 5mM EDTA, 1% Triton-X 100, 2 mM orthavanadate, 50mM sodium fluoride, 20mM phenylarsine oxide, 1mM PMSF, 10µg/ml leupeptin, 10µg/ml aprotonin, 10mM sodium molybdate. Cells were scraped from the surface of the flasks and transferred into Eppendorf tubes, then spun for 6min at 13 000rpm at 4°C. The protein content of the supernantant was determined using a standard Bio-Rad assay according to manufactures guidelines. Protein extractions were stored at –20°C (or –70°C for long term storage).

(ii) Western blotting

Protein lysates (100µg) were resolved on 7.5-12% SDS-PAGE. Aliquots of equal concentration were made of protein samples, their volumes equalised and 1x loading buffer (5x buffer: 125mg Tris Base, 1.25g SDS, 6.25ml β-mercaptoethanol, 12.5ml glycerol, 417µl bromophenol blue solution, made up to a total volume of 25ml with H_2O) added. Samples were denatured for 6min at 95°C and loaded onto a 7.5-12% polyacrylamide gel. Gels were run at 60mA for 35 min followed by 35mA for 4h. Proteins were then electrophoretically transferred onto permeablised Immobilon-P membranes at 30V, 4°C overnight. After transfer, membranes were blocked and probed with primary antibody overnight at 4°C. Immunoreactive bands were detected using chemiluminescent Western Blotting Kits (ECL or Super Signal West Femto Maximum Sensitivity Substrate) and photographic paper. Standard molecular weight markers were used to estimate protein size. Antibodies are listed in table 4.2.

Table 4.2 Antibodies used for Western blotting

Primary antibody	**Type**	**Raised in**	**Dilution**	**Supplier details**
PR (PgR: Clone hpRa2 +hPRa3)	IgG_1/κ	Mouse	1µg/ml	Neomarkers: Ab-8 # MS-298
ERα (F-10)	Monoclonal IgG_{2a}	Mouse	1µg/ml	SANTA CRUZ BIOTECHNOLOGY, INC #sc-8002
SRC-3(AIB-1)	IgG1	Mouse	2µg/ml	Affinity Bioreagents: #MA1-845
RIP140	Polyclonal IgG	Rabbit	3µg/ml	Affinity Bioreagents: #PA1-841
REA	Polyclonal IgG	Rabbit	1µg/ml	Upstate #07-234
SMRT	Polyclonal IgG	Rabbit	2µg/ml	Upstate #06-891
NCoR	Polyclonal IgG	Rabbit	2µg/ml	Upstate #06-892
SRC-1	IgG_1/κ	Mouse	1µg/ml	UpState: #05-522
SRC-2 (TIF2)	IgG1	Mouse	1µg/ml	BD Biosciences #610984
Actin	IgM	Mouse	1/120,000	CALBIOCHEM@ #CP01
Secondary antibody				
Anti-Mouse	Polyclonal IgG HRP	Donkey	1/1000 (used for PgR, SRC-1/2/3)	Abcam #ab7061
Anti-Rabbit	Polyclonal IgG HRP	Sheep	1/1000 (used for SMRT, NCoR) 1/1500(used for REA, RIP140)	Abcam #ab6795
Anti-Mouse	Polyclonal IgM HRP	Goat	1/2000 (used for actin)	CALBIOCHEM@ #401225

4.2.4 Chromatin immunoprecipitation

(i) ChIP assay

a. Cell Harvesting

MCF-7 cells were seeded in complete DMEM with 10% FCS for 24 h. Cells were then washed twice in PBS and media added to reduced DMEM containing 5% DCC for at least 48 h. LCC-1, LCC-2 and LCC-9 cells were immediately seeded in reduced DMEM containing 5% DCC. All cells were plated into 92mm petri dishes and grown to 85-90% confluence. Two dishes each containing approximately $1x10^6$ were set up for each timepoint. Following the treatment with 10^{-9} M E_2 at various intervals, cells were cross-linked with 1% formaldehyde for 10min at 37°C and fixed with 0.125M glycine on a rocker for 10min at RT. Cells were then washed with ice-cold PBS before collection into PBS (1ml/plate) containing protease inhibitors tablets (1 tablet/10ml buffer). Samples were kept on ice at all times from this point. To prepare for lysis, samples were transferred into 1.5ml Eppendorf tubes and centrifuged for 4min at 4°C and 2000rpm. The supernatant was carefully aspirated and pellets resuspended in lysis buffer (50mM TrisHCl pH8.1, 10mM EDTA, 1%SDS), 200µl per dish containing protease inhibitor tablets as above. Duplicate dishes of the same time point were now combined and left for 10min. Samples were then sonicated 3x10sec at 4Aµ to shear DNA to appoximately 500-1000bp fragments followed by centrifugation for 15 min at 4°C and 13 000rpm. Supernatants were collected into 4x100µl aliquots, the equivalent of 4 immunoprecipitations and snap frozen at -70°C until use for immunoprecipitation.

b. Verification of DNA fragment size

After sonication and centrifugation as described above, 5µl 4M NaCl was added to 100µl chromatin mixture (one aliquot of the sonicated sample) and heat treated at 65°C for 4h. To precipitate DNA fragments,100µl commercially available phenol-chloroform solution was added, the sample vortexed for 10sec and spun at 13 000rpm for 5min at 4°C. The upper phase was transferred to a fresh tube and 10µl 3M NaAcetate/ 220µl ethanol added. The sample was vortexed and placed in the -20°C freezer for a minimum of 30min. DNA fragments were then pelleted by centrifugation for 5min at 13 000rpm and washed with 1ml of 70% ethanol at RT.

The air dried pellet was resuspended in 50µl TE buffer. Fragments were visualized on a 1.5% agarose gel.

c. Immunoprecipitation

One aliquot per time point was diluted 10fold in dilution buffer (16.7mM TrisHCl pH8.1, 1.2mM EDTA, 167mM NaCl, 1.1%Triton, 0.01%SDS). A portion (3% of input) of this chromatin solution was set aside for quantification of DNA amount present in each sample. To preclear the chromatin solution and reduce non-specific background, 50µl Protein G-Agarose Beads per sample were washed 3x in 1ml dilution buffer. To the beads, 1µg/reaction anti-rabbit IgG and 2µg/ reaction salmon sperm DNA was added, the mixture made up to 100µl with dilution buffer and aliquoted into Eppendorf tubes. Samples were transferred to the bead mix and rotated at 15rpm for at least 3h at 4°C. Beads were then pelleted briefly and the supernatant transferred to newly prepared beads (also 50µl/reaction) containing salmon sperm DNA and incubated with specific antibodies on a rotator at 12rpm at 4°C overnight. Antibodies are listed in table 4.4.

d. Recovery and PCR

After incubation, beads were washed sequentially with TSE I (20mM Tris HCl ph8.1, 2mM EDTA, 150mM NaCl, 1% Triton X-100, 0.1% SDS), TSE II (20mM Tris HCl ph8.1, 2mM EDTA, 500mM NaCl, 1% Triton X-100, 0.1% SDS) and Buffer III (10mM Tris HCl ph8.1, 1mM EDTA, 0.25M LiCl, 1% IgePal-CA630, 1% deoxycholic Acid). Bead complexes were then eluted on a rotater at RT with 250µl freshly prepared elution buffer (1%SDS,0.1M $NaHCO_3$) for 30min followed by 15min in 200µl and the supernatants collected into new Eppendorf tubes. To reverse formaldehyde cross-linking, each sample (as well as input samples) was heat treated at 65°C for 6h in 25µl 4M NaCl and 45°C for 1h with 32µl of a proteinase K cocktail (10µl 0.5M EDTA, 20µl 1M Tris-HCl pH6.5, 2µl 10mg/ml proteinase K). DNA fragments were purified using QIAquick Spin Kit columns according to manufacturers instructions and amplified using 2µl of each sample and the QuantiTect™SYBR®Green system (Qiagen cat#204242) . PCR conditions were: initial activation of 95°C for 15min followed by 45cycles of 94°C for 15sec, 55°C for 30sec, 72°C for 30sec; and a final extension of 72°C for 5min. Primers are listed in table 4.3.

(ii) ChIP assay using tumour material

The protocol for this assay is largely based upon a protocol published by Dr. Peggy Farnham's laboratory at the UC Davis Genome Center in California, USA (http://genomecenter.ucdavis.edu/farnham/protocol.html). Tumour tissue was thawed and minced with a razor blade. The tissue was transferred into tubes with srcew cap lids and a small amount of tissue culture media added. To cross-link, a final concentration of 1% formaldehyde was added to each sample and the sample rotated at RT for 15min. A final concentration of 0.125M glycine was added to stop the cross-linking and rotated for a further 5min. All steps were carried out on ice from here on forward. Samples were now centrifuged for 4min at 4000rpm, the media decanted and ice-cold PBS added to wash the tissue. After subsequent centrifugation and resuspension of the pellet in 1ml of ice-cold PBS, the tissue was liquified for 2x 10sec using a dounce homogenizer. Tissue was pelleted at 5000rpm for 4min and resuspended in 1ml lysis buffer (50mM TrisHCl pH8.1, 10mM EDTA, 1%SDS) containing protease inhibitors (1tablet/10ml). Samples were allowed to lyse for 15min on ice before sonication at 4Aμ for 3x10sec to fragment chromatin into 500-1000bp pieces. After centrifugation at 13,000rpm for 15min at 4°C, the supernatant was aliquoted into new Eppendorf tubes containing approximately 0.03 tumour tissue. Chromatin fractions were snap frozen at -70°C until use for immunoprecipitation as described in in chapter 4.2.3: i, b and PCR in chapter 4.2.3; i c.

Table 4.3 Primers used for ChIP PCR

Target DNA	NCBI number	Primer sequence	Product size
pS2 promoter forward	X05030	F5'GACGGAATGGGCTTCATGAGC3'	353bp
pS2 promoter reverse		R5'CTGAGACAATAATCTCCACTG3'	
pS2 distal forward	NT030188	F5'CTT GCC TCT GCA TTC TCT CC3'	185bp
pS2 distal reverse		R5'GAG TTT GGC CTC CCA CAT TA3'	

pS2 promoter primers were designed and are published by Shang, Y. *et al.* 2000.

Table 4.4: Antibodies used for ChIP

Primary antibody	**Type**	**Raised in**	**Dilution**	**Supplier details**
Anti-Hyperacetylated Histone H4 (Penta)	KLH-conjugated peptide corresponding to amino acids 2-19	Rabbit	5µl/ rxn	Upstate: # 06-946
Anti-acetyl-Histone H4 (Lys 12)	Ovalbumin-conjugated, synthetic peptide corresponding to aa 7-18	Rabbit	5µl/ rxn	Upstate: # 07-323
Anti-dimethyl – Histone H4 (Lys 20)	Polyclonal IgG	Rabbit	5µl/ rxn	Upstate: # 07-367
Anti-acetyl-histone H4	KLH-conjugated peptide corresponding to amino acids 2-19	Rabbit	5µl/ rxn	Upstate: # 06-866
SRC-3 (RAC-3 C-20) NCoA-3	Polyclonal IgG	Goat	5µl/ rxn	SANTA CRUZ BIOTECHNOLOGY, INC #sc-543
SRC-3 (MA1-845)	IgG1	Mouse	5µl/ rxn	ABR Affinity Bioreagents™ # MA1-845
SRC-3 (05-490) Anti-ACTR/AIB1	IgG	Mouse	5µl/ rxn	Upstate #05-490
SRC-1	IgG_1/κ	Mouse	5µl/ rxn	Upstate: #05-522
ERα HC-20	IgG	Rabbit	5µl/ rxn	Santa Cruz: #sc-543

Chapter 5: References

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